REGULATION OF TLR9-INDUCED INNATE IMMUNE RESPONSES IN SHEEP PEYER'S PATCHES

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ABSTRACT

One of the fundamental questions in mucosal immunology is how the intestine maintains tolerance to food antigens and commensal flora, and yet it is capable of mounting immune responses to pathogens. Peyer's patches (PP) are lymphoid aggregates that are found in the small intestine and are the primary sites where adaptive immune responses are initiated in the intestine. An understanding of how PP cells regulate innate immune responses may provide information on how immune responses are regulated in the intestine. The toll-like receptors (TLRs) are a family of pattern recognition receptors (PRR) which provide a sensory mechanism for the detection of infectious threats. TLR9 recognizes bacterial DNA or synthetic CpG oligodeoxynucleotides (ODN). Cells that express TLR9 when stimulated with CpG ODN proliferate and produce Th1-like pro-inflammatory cytokines and upregulate co-stimulatory molecules. Because the intestine is constantly exposed to bacterial DNA from commensal flora, immune cells from the gut must have evolved mechanisms to modulate responses to TLR9 stimulation to prevent responses to harmless bacteria. Our hypothesis is that innate immune responses to the TLR9 agonist CpG ODN in Peyer's patches (PP) are attenuated compared to other tissues such as blood or lymph nodes. This is due to local regulatory mechanisms unique to the intestinal microenvironment.

We conducted a number of experiments to test this hypothesis. We initially assessed the immunostimulatory activity of three available classes of CpG ODN in lymph nodes (LN), peripheral blood mononuclear cells (PBMC) and PP since this had not been done in ruminants. We found that CpG ODN induced strong IFNα, IFNγ, IL-12, lymphocyte proliferation and NK-like activity in LN and PBMC. In contrast, these responses were significantly less in PP stimulated with CpG ODN. We wondered whether the reduced responses of PP cells to CpG ODN were unique to the TLR9 agonist. For this reason we tested responses of cells from these tissues to poly (I:C), LPS, and single-stranded RNA, which are agonists for TLR3, TLR4, and TLR7/8 respectively. Additionally, we tested combinations of TLRs since others have reported that multiple TLR agonists may induce synergistic responses. All TLR agonists or their combinations either failed to induce detectable responses or the responses were

significantly less in PP compared to other tissues. Thus we concluded that PP cells responses to TLR stimulation were attenuated. In all tissues tested, there were no synergistic responses (IFNα, IFNγ and lymphocyte proliferation) following stimulation with combinations of agonists. However, there was inhibition of PBMC responses when TLR7/8 agonists were combined with CpG ODN (TLR9 agonist). Importantly, TLR7/8 agonists reduced the CpG-induced proliferative responses in purified blood B cells. Interestingly, ovine B cells constitutively expressed TLR7/8 and TLR9 mRNA, suggesting the potential for cross-talk between the receptors.

Interestingly, cell from all isolated tissues [ileal PP (IPP), jejunal PP (JPP), mesenteric LN (mLN) and PBMC] expressed similar levels of TLR9 mRNA, suggesting that the reduced responsiveness to CpG ODN stimulation in PP was not due to a lack of TLR9 expression.

Surprisingly, we observed that PP cells spontaneously secreted significant amounts of the immunoregulatory cytokine IL-10. Furthermore, we confirmed that $CD21^+$ B cells were the source of the IL-10. We then examined the role of IL-10 in regulating IFN and IL-12 responses in PP. Neutralization of IL-10 resulted in a significant increase in the numbers of CpG-induced IFNα-secreting cells detected and in IFNγ and IL-12 production by PP cells (both follicular and interfollicular lymphocytes). Similarly, depletion of the $CD21^+$ B cells resulted in significant increases in IFNα, IFNγ and IL-12 responses. These observations support the conclusion that IL-10-secreting PP $CD21^+$ B cells suppress innate immune responses in PP. Further characterization by flow cytometry revealed that these cells were $CD1b^-CD5^-CD11c^-$ $CD72^+CD21^+$ IgM^+ B cells. We have proposed that these IL-10-secreting PP $CD21^+$ B cells are a novel subset of regulatory B cells (B_{regs}).

Finally, we examined the capacity of IL-10 secreting B cells (B_{regs}) to respond to CpG ODN. To achieve this, we compared $CD21^+$ B cells from blood and JPP. Unlike blood $CD21^+$ B cells, $CD21^+$ B cells from JPP proliferated poorly in response to CpG ODN. Moreover, PP $CD21^+$ B cells, unlike blood $CD21^+$ B cells, do not secrete IgM or IL-12 in response to CpG stimulation, although both PP and blood $CD21^+$ B cells express similar level of TLR9 mRNA. Neutralization of IL-10 did not enhance CpG-induced proliferative responses in PP $CD21^+$ B cells. Thus IL-10 does not play a direct

role in the hyporesponsiveness of PP $CD21^+$ B cells to CpG ODN. To further explore the mechanism by which PP B_{regs} fail to respond to CpG ODN stimulation, we used a kinome analysis to determine whether the TLR9 pathway was functional in PP B_{regs} compared to blood $CD21^+$ B cells. We observed that peptides representing critical adaptor molecules downstream of TLR9 such as IRAK1, TAK1, Casp8, p-38 MAPK, JNK, FOS, IKKα, NFκB-p65 were not phosphorylated in JPP $CD21^+$ B cells following CpG ODN stimulation. However, in blood $CD21^+$ B cells stimulated with CpG ODN, the same peptides on the array were all highly phosphorylated leading to a functional TLR9 signaling pathway. Thus PP B_{regs} have evolved mechanisms by which the TLR9 signaling pathway is not activated following exposure to the TLR9 agonist, CpG ODN.

In conclusion, we clearly demonstrated that TLR9-induced responses in cells from PP are significantly attenuated. This is a consequence of PP $CD21^+$ B cells (B_{regs}) that spontaneously secrete IL-10, which in turn "conditions" an anti-inflammatory environment in this tissue leading to poor cytokine responses to the TLR9 agonist, CpG ODN. Additionally, we show that B_{regs} are unresponsiveness to TLR9 stimulation. This unresponsiveness is due to regulatory mechanisms in B_{regs} leading to a dysfunctional TLR9 signaling pathway. These may represent strategies by which PP dampen innate responses to pathogen-associated molecular patterns (PAMPs) in intestinal immune tissues to maintain intestinal immune homeostasis. These conclusions are consistent with our hypothesis that TLR responses in PP cells are attenuated, and this is due to B cell-mediated regulatory mechanisms that are unique to the intestinal microenvironment.

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TABLE OF CONTENTS

PERMISSION TO USE ..... **i**
ABSTRACT ..... **ii**
ACKNOWLEDGEMENTS ..... **v**
TABLE OF CONTENTS ..... **vi**
LIST OF TABLES ..... **xii**
LIST OF FIGURES ..... **xiii**
LIST OF ABBREVIATION ..... **xv**
CHAPTER 1: LITERATURE REVIEW **1**
1.1 Innate Immune System **1**
1.1.1 Pattern recognition receptors (PRR) 2
1.1.2 TLR 3
1.1.3 TLR9 and CpG motif recognition 7
1.2 Innate Immune responses induced by CpG ODN **8**
1.2.1 Dendritic cells responses to CpG ODN 9
1.2.2 B cell responses to CpG ODN 10
1.2.3 Other immune cells stimulated by CpG ODN 11
1.3 Simultaneous multiple TLR stimulation **12**
1.3.1 Multiple TLR stimulation 12
1.4 Overview of the Mucosal Immune system **13**
1.4.1 Gut-associated lymphoid tissues 14
1.4.2 Peyer's patches 14
1.4.3 Distribution and function of PP in sheep 15
1.4.4 Sheep PP B cells 16
1.4.5 Mucosal DC 17
1.4.6 Mechanisms leading to intestinal homeostasis 18
1.4.6.1 Downregulation of TLR and their adaptors on immune cells 18
1.4.6.2 Inhibitors in TLR signaling pathways 19
1.4.6.3 Cellular localization of TLR9 19
1.4.6.4 Th2 cytokine bias 20

1.4.6.5 Immunoregulatory cytokines (IL-10, TGFβ) 21
1.5 B lymphocytes 21
1.5.1 TLR9 in B cell activation 21
1.5.2 Cytokine secreting B cells 23
1.5.2.1 Effector B cells 23
1.5.2.2 Regulatory B cells 24
CHAPTER 2: RATIONALE, HYPOTHESIS, OBJECTIVES AND AIMS 25
2.1 Rationale and hypothesis 25
2.2 Overall Objective 26
2.3 Aims 26
CHAPTER 3: INNATE IMMUNE RESPONSES INDUCED BY CLASSES OF CpG ODN IN OVINE LYMPH NODE AND BLOOD MONONUCLEAR CELLS (as published in Booth et al., 2007; Vet. Immuno.Immunopath.) 27
3.1 Abstract 27
3.2 Introduction 28
3.3 Materials and methods 31
3.3.1 Oligodeoxynucleotides 31
3.3.2 Animals 31
3.3.3 Isolation of PBMC and LN 32
3.3.4 Tissue culture conditions 41
3.3.5 ELISA for IFNα, IFNγ and IL-12 33
3.3.6 Cytotoxicity assay 34
3.3.7 Lymphocyte Proliferative responses (LPR) 34
3.3.8 Statistical analysis 35
3.4 Results 36
3.4.1 Effect of CpG ODN class and concentration in PBMC and LN 36
3.4.1.1 IFNα induction in LN and PBMC 36
3.4.1.2 IFNγ induction in LN and PBMC 37
3.4.1.3 Proliferative responses in LN and PBMC 40

3.4.2 IL-12 induction in LN and PBMC....41
3.4.3 NK-like cytotoxicity in LN and PBMC....44
3.5 Discussion....46
3.6 Conclusion....48
CHAPTER 4: SHEEP PEYER'S PATCH CELLS RESPONSES TO TLR AGONISTS WERE ATTENUATED AND TLR7/8 AGONISTS DOWNREGULATE CpG-INDUCED RESPONSES IN BLOOD (As submitted to Developmental and Comparative Immunology.) 49
4.1 Abstract....49
4.2 Introduction....51
4.3 Materials and methods....54
4.3.1 TLR agonists and Animals....54
4.3.2 Isolation of PBMC, JPP, IPP and LN....54
4.3.3 Magnetic activated cell sorting (MACS)....55
4.3.4 Tissue culture conditions and stimulation with TLR agonists....55
4.3.5 ELISA for IFNα, IFNγ, IL-10 and IL-12....56
4.3.6 Caspase assay....57
4.3.7 Lymphocyte Proliferative responses....57
4.3.8 Quantitative RT-PCR....58
4.3.9 Statistical analysis....59
4.4 Results....60
4.4.1 PP respond poorly to TLR agonists compared to LN and PBMC....60
4.4.2 PP express similar levels of TLR mRNA to mLN and PBMC....66
4.4.3 ORN suppress the CpG-induced innate immune responses in PBMC, mLN and purified B cells....67
4.4.4 Imiquimod, another TLR7/8 agonist also suppresses CpG-induced responses in a dose dependent manner....70
4.4.5 Reduction of CpG-induced responses by TLR7/8 agonists is not associated with induction of cell death or the anti-inflammatory cytokine IL-10....71

4.4.6 Suppression of CpG-induced responses was not due to preferential cellular uptake of the TLR7/TLR8 agonists 73

4.5 Discussion 74

4.6 Conclusion 78

CHAPTER 5: A NOVEL REGULATORY B CELL POPULATION IN SHEEP PEYER'S PATCHES SPONTANEOUSLY SECRETES IL-10 AND DOWNREGULATES TLR-9-INDUCED IFNα RESPONSES (As published in Booth et al., 2009; Mucosal Immunology.) 79

5.1 Abstract 79

5.2 Introduction 80

5.3 Materials and methods 82

5.3.1 Oligodeoxynucleotides 82

5.3.2 Animals 82

5.3.3 Isolation of PBMC, JPP, IPP and LN 82

5.3.4 Tissue culture conditions 83

5.3.5 ELISA for IFNα, IFNγ, IL-10 and IL-12 84

5.3.6 IFNα ELISPOT Assay and neutralization of IL-10 85

5.3.7 Magnetic activated cell sorting (MACS) 85

5.3.8 Quantitative RT-PCR 86

5.3.8.1 mRNA isolation and cDNA synthesis 86

5.3.8.2. Real-time RT-PCR 87

5.3.9 Statistical analysis 88

5.4. Results 89

5.4.1 IFNα and IFNγ secretion is reduced in Peyer's patch cells stimulated with CpG ODN 89

5.4.2 Reduced cytokine responses of PP cells are not due to suboptimal CpG ODN concentration 91

5.4.3 IL-12 secretion is also reduced in PP stimulated with CpG ODN 94

5.4.4 Peyer's patches have a lower frequency of IFNα producing cells 96
5.4.5 Peyer's Patches constitutively express TLR9 mRNA. 97
5.4.6 Role of IL-10 in regulation of CpG-induced responses in PP.....100
5.4.6.1 Kinetics of IL-10 production in PP 100
5.4.6.2 IL-10 inhibits IFNα production in PP 102
5.4.7 Peyer's patch $CD21^+$ B cells are the major source of the IL-10 103
5.4.8 $CD21^+$ PP B cells inhibit CpG-induced responses in JPP. 104
5.4.9 Characterization of JPP $CD21^+$ B cells by FACs analysis 106
5.5 Discussion 108
5.6 Conclusion 112
CHAPTER 6: TLR9 SIGNALING FAILURE RENDERS PEYER'S PATCH REGULATORY B CELLS UNRESPONSIVE TO CpG ODN STIMULATION(As submitted to Mucosal Immunology.) 113
6.1 Abstract 113
6.2 Introduction 114
6.3 Materials and methods.....116
6.3.1 Oligonucleotides and Animals 116
6.3.2 Isolation of PBMC, JPP, and lymph node cells (LN) and tissue culture conditions.....116
6.3.3 ELISA for IL-10, IL-12 and IgM 117
6.3.4 Cell purification using magnetic activated cell sorting (MACS) 117
6.3.5 Caspase assay 117
6.3.6 Lymphocyte Proliferative responses (LPR) 118
6.3.7 Quantitative RT-PCR 118
6.3.8 Kinome Analysis 119
6.3.9 Statistical analysis 120
6.4. Results.....121
6.4.1 PP cells proliferate poorly in response to CpG ODN stimulation.....121
6.4.2 PP $CD21^+$ B cells fail to secrete significant IgM upon stimulation with CpG ODN. 125

6.4.3 PP CD21$^+$ B cells express high level of TLR9 mRNA 126
6.4.4 PP and blood CD21$^+$ B cells express similar levels of apoptotic markers ... 127
6.4.5 PBMC CD21$^+$ B cells but not PP CD21$^+$ B cells secrete IL-12 128
6.4.7 Unresponsiveness of PP CD21$^+$ B cells to CpG ODN is not due to IL-10. . 129
6.4.8 Failure of TLR9 signaling in PP CD21$^+$ B cells……...148
6.5 Discussion 135
6.6 Conclusion: 136
CHAPTER 7: GENERAL DISCUSSION 137
7.1 Intestinal homeostasis 137
7.2 B_{regs} : Characteristic and regulation of TLR9 signaling pathway 139
7.3 B_{regs}: Differentiating between commensals and pathogens 140
7.3.1 Model 141
7.4 Significance of B_{regs} in Peyer's patches 144
CHAPTER 8: CONCLUSION 145
8.1 Overall Conclusion 145
8.2 Future work 145
CHAPTER 9: REFERENCES 146
9.1 References: 146

LIST OF TABLES

Table 1.1: TLR members recognizing PAMPs……..4

Table 1.2: Differences between sheep IPP and JPP…….....16

Table 3.1: A-, B- and C-class CpG ODN sequences and backbone structure….....31

Table 6.1: Phosphorylation state of blood and PP $CD21^{+}$ B cells……..131

LIST OF FIGURES

Fig. 1.1: General TLR pathway. 6
Fig. 1.2: Diagramatic representation of Peyer's patch. 15
Fig. 3.1 A-B: IFNα secretion by ovine PBMC and LN.. 1
Fig. 3.2 A-B: IFNγ secretion by ovine PBMC and LN. 1
Fig. 3.3 A-B: Proliferative responses by ovine PBMC and LN. 1
Fig. 3.4 A-B: IL-12 responses in ovine PBMC and LN.. 43
Fig. 3.5 A-B: NK-like cytotoxicity responses in ovine PBMC and LN. 45
Fig. 4.1A-D: IFNα responses in ovine IPP, JPP, mLN and PBMC 60
Fig. 4.2 A-D: IFNγ responses in ovine IPP, JPP, mLN and PBMC. 61
Fig. 4.3 A-D: Proliferative responses in ovine IPP, JPP, mLN and PBMC 62
Fig. 4.4 A-D: IgM responses in ovine IPP, JPP, mLN and PBMC 1
Fig. 4.5 A-D: Proliferative responses in IPP, JPP, mLN and PBMC with TLR agonists 65
Fig. 4.6 A-B: Expression of TLR7 and TLR8 in IPP, JPP, mLN and PBMC.. 66
Fig. 4.7: Expression of TLR9 in IPP, JPP, mLN and PBMC.. 67
Fig. 4.8 A-B: Simultaneous cell activation with ORN and CpG ODN 1
Fig. 4.9 A-B: ORN suppress the CpG-induced IgM responses 69
Fig. 4.10: Suppression of CpG-induced proliferative responses with ORN. 69
Fig. 4.11 A-B: Suppression of CpG-induced proliferative responses is dose-dependent. 1
Fig. 4.12 A-B: IL-10 responses by ovine PBMC and purified B cells.. 72
Fig. 4.13: Caspase 3/7 responses by ovine purified PBMC-CD21$^+$ B cells 73
Fig. 4.14 A-B: Proliferative responses by PBMC (A) and purified B cells (B).. 1
Fig. 5.1 A-B: IFNα and IFNγ secretion by ovine IPP, JPP and LN cells.. 90
Fig. 5.2 A-D: IFNα secretion by ovine IPP, JPP, mLN cells and PBMC. 92
Fig. 5.3 A-D: IFNγ secretion responses by ovine IPP, JPP, mLN cells and PBMC.....93
Fig. 5.4 A-D: IL-12 secretion by ovine IPP, JPP, mLN cells and PBMC 95
Fig. 5.5 A-D: Frequency of IFNα producing cell in IPP, JPP, PBMC, and mLN. 1
Fig. 5.6: Frequency of IFNα producing cells compared to IPP. 1

Fig. 5.7 A-B: TLR9 mRNA expression in ovine tissues. 99
Fig. 5.8 A-D: Kinetics of IL-10 secretion by IPP, JPP, mLN and PBMC.....101
Fig. 5.9: Kinetics of IL-10 secretion by IPP, JPP, mLN and PBMC unstimulated. 102
Fig. 5.10 A-C: IFNα producing cells responses in ovine IPP, JPP and PBMC.....103
Fig. 5.11 A-F: PP $CD21^+$ B cells secrete IL-10 spontaneously 1
Fig. 5.12 A-C: $CD21^+$ B cell inhibit CpG-induced responses. 105
Fig. 5.13: Isolated JPP $CD21^+$ B cells with purity of 97.2% co-labelled 1
Fig. 5.14: Isolated PBMC $CD21^+$ B cells with purity of 90.2% co-labelled 1
Fig. 6.1: Lymphocyte proliferative responses in IPP, JPP, mLN and scLN.....121
Fig. 6.2 A-D: Proliferative responses by IPP, JPP, mLN and PBMC.. 123
Fig. 6.3 A-B: Proliferative responses in isolated $CD72^+$ and $CD72^-$ cell fractions 124
Fig. 6.4 A-F: Proliferative responses in purified $CD21^+$ and $CD21^-$ cell fractions. 1
Fig. 6.5 A-B: IgM responses in isolated $CD21^+$ and $CD21^-$ cell fractions 1
Fig. 6.6: TLR9 expression in isolated $CD21^+$ and $CD21^-$ cell fractions.. 127
Fig. 6.7: Caspase 3/7 expression in PP-$CD21^+$ and PBMC-$CD21^+$ cell fractions. 128
Fig. 6.8 A-B: IL-12 secretion in $CD21^+$ cell fractions from PBMC and JPP. 129
Fig. 6.9 A-B: Neutralization of IL-10 in PP B regulatory cells... 130
Fig. 6.10: Phosphorylation state of adaptor molecules in TLR9 signaling pathway in CD21+ B cells from blood and JPP……133
Fig. 7.1: Model demonstrating the potentioal role of PP Bregs in regulating TLR responses to commensal bacteria……143

LIST OF ABBREVIATION

a.a	Amino acid
Ab	Antibody
AIS	Adaptive immune system
am DKO	B cells deficient TCRα double KO
ANOVA	One-way analysis of variance
AP	Alkaline phosphatase
AP-1	Activator protein 1
APC	Antigen presenting cells
APRIL	A proliferation-inducing ligand
BAFF	B cell activating factor
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BCR	B cell receptor
bIFN-γ	Recombinant bovine IFNγ
bIFN-α	Bovine IFNα
BLAST	Basic Local Alignment Search Tool
Be cells	B-effector cells
B_{regs}	B regulatory cells
BSA	Bovine serum albumin
BTK	Bruton's tyrosine Kinase
bZIP	Basic region-leucine zipper
c.p.m.	Counts per minute
CARD	Cytoplasmic caspase-recruiting domain
cDC	Conventional DC
cDNA	Complimentary Deoxyribonucleic acid
CFA	Complete Freund's Adjuvant
ConA	Concavalin A
CpG	Cytosine phosphate Guanosine
Ct	Cycle threshold
DC	Dendritic cells

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOTAP	N-(2,3-Dioleoyloxy-1-propyl) trimethylammonium methyl sulfate
dsRNA	Double stranded RNA
DTT	Dithiothreitol
DUBA	Deubiquitinating enzyme A
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene di-amine tetra-acetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
FACs	Fluorescence assisted cell sorting
FADD	FAS-associated death domain
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum
FO B cells	Follicular B cells
FOS	Proto-oncogene protein
Foxp3	Forkhead box P3
G^{+}ve	Gram-positive
GALT	Gut-associated lymphoid tissues
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine Cytosine
GIPLs	Glycosylphosphatidylinositol lipids
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G^{-}ve	Gram-negative
HCl	Hydrochloric acid
HEPES	[N(-2-hydroxyethyl)] piperazine-n'-[2-ethane sulfonic acid]
hrs	Hours
IBD	Inflammatory bowel disease
ICAM-1	Inter-cellular adhesion molecule 1

IEC	Intestinal epithelial cells
IFN	Interferon
IgA	Immunoglobulin A antibodies
IIS	Innate immune system
IkBα	Inhibitor of NFκB
IKK	Inhibitor of NFκB kinase
IPP	Ileal PP
IPS	Interferon-β promoter stimulator
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
ISC	IFNα-secreting cells
IU	International unit
JNK	JUN N-terminal kinase
JPP	Jejunal PP
JUN	Proto-oncogene c-jun
KCl	Potassium chloride
KO	Knock-out
LN	Lymph nodes
LNC	Cervical lymph node
LPR	Lymphocyte Proliferative Responses
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
M cell	Microfold cell
MACS	Magnetic-activated cell sorting
MAL	MyD88-adaptor-like
MALT	Mucosal-associated lymphoid tissues
MAMP	Microbial-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation-associated gene 5
mDC	myeloid DC

MEM	Minimum essential medium
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
mLN	Mesenteric lymph nodes
mM	Millimolar
MMTV	Mouse mammary tumor virus
Mϕ	Macrophages
mRNA	Messenger Ribonucleic acid
MyD88	Myeloid differentiation 88
MZ	Marginal zone
NIH	National Institutes of Health
NK	Natural killer
NKT	Natural Killer T cell
NLR	Nucleotide-binding oligomerization domain (NOD)- like receptors
nM	Nanomole
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotides
Oligo dt	Oligodeoxythymidylic acid
ORN	Oligoribonucleotides
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBSA	Phosphate-buffered saline calcium and magnesium free
PCR	Polymerase Chaín reaction
pDC	Plasmacytoid dendritic cells
PE	phycoerythrin
PKACa	cAMP dependent protein kinase alpha
PMSF	Phenymethylsufonyl fluoride
PNPP	P-nitrophenyl phosphate

PO	Phosphodiester
Poly dI-dC	Polydeoxyinosinic-deoxycytidylic acid
Poly U	Poly (U) polymerase
PP	Peyer's patches
PPARγ	Peroxisome proliferator-activated receptor-γ
pre-DC	Precursor DC
PRR	Pattern recognition receptors
PS	Phosphorthothioate
qRT-PCR	Quantitative reverse transcriptase PCR
RA	Rheumatoid arthritis
rHuIL-12	Recombinant human IL-12
RIG-1	Retinoic acid-inducible gene 1
RLR	RIG-like receptors
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
SARM	Sterile α-and armadillo-motif-containing protein
scLN	Superficial cervical lymph node
SED	Subepithelial dome
SHP-1	SH2 domain-containing protein phosphatase 1
SIGIRR	Single immunoglobulin IL-1R-related molecule
sIgM	Surface IgM
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
SOCS1	Suppressor of cytokine signaling 1
ssRNA	Single stranded RNA
TAB1	TAK1 binding protein 1
TAB2	TAK1 binding protein 2
TAK1	Transforming growth factor β-activated kinase
TBM	Tingible body macrophages
TBST	Tris buffered-saline/0.01% Tween 20

TBST-g	TBST/0.5% gelatin
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th1	T helper 1 cells
Th2	T helper 2 cells
Th3	TGFβ1-producing regulatory T cells
TICAM1	TIR domain containing adaptor protein inducing IFNβ
TICAM2	TRIF-related adaptor molecule
TIR	Toll/IL-1R
TIRAP (MAL)	MyD88-adaptor-like
TLR	Toll-like receptors
TOLLIP	Toll-interacting protein
Tr1	IL-10-producing regulatory T cells
TRAF	Tumour-necrosis factor receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM(TICAM2)	TRIF-related adaptor molecule
T_{regs}	Regulatory T cells
TRIF(TICAMI)	TIR domain-containing adaptor protein-inducing IFNβ
TSLP	Thymic stromal lymphopoietin
UBC13	Ubiquitin-conjugating enzyme 13
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1
WWW	World wide web
β-actin	Beta actin

CHAPTER 1: LITERATURE REVIEW

1.1 Innate Immune System

Vertebrate immune defenses consist of two complementary systems, namely the innate immune system (IIS) and adaptive immune system (AIS). Though complementary, the two immune systems have distinct attributes including recognition of microbial molecules, the cells involved and their mechanisms of action. The AIS has two main effector cell populations, B and T lymphocytes, which can recognize single epitopes expressed on foreign antigens when presented in the context of MHC-antigen complexes. The hallmarks of the AIS are specificity, repertoire diversity and memory [1].

Innate immunity is the focus of the work described in this thesis. The IIS has been described as the first line of immune defense against pathogens. Originally, it was thought that IIS were completely non-specific but it is now known that the IIS can indeed discriminate between self-antigens and a variety of pathogens. For example, the innate immune system can recognize lipopolysaccharide (LPS) as foreign by using innate sensors such as Toll like receptor 4 (TLR4). Thus it plays a critical role in the detection of pathogens [2]. The IIS utilizes limited germ-line-encoded receptors called pattern recognition receptors (PRR) [3, 4]. There are various families of PRR such as the Toll-like receptors (TLRs), Retinoic acid-induced gene (RIG)-like receptors (RLR), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), mannose receptors, scavenger receptors, and natural killer (NK)-activating receptors (CD16, NKG2D). The cells that are involved in IIS include dendritic cells (DC), natural killer cells (NK), macrophages (Mϕ), neutrophils, basophils, eosinophils, epithelial cells and mast cells although each of these are involved in the AIS response. For a long time, the IIS has been described as an evolutionarily ancient system that helps in the rapid clearance of pathogens during the early phase of infection. There is now overwhelming evidence that IIS is not only involved in the early clearance of pathogens but it also initiates and shapes the development of the adaptive responses [2, 5]. In turn, the AIS

has been shown to influence the innate responses [6]. Thus, for pathogen clearance, coorperation between the IIS and AIS is necessary.

1.1.1 Pattern recognition receptors (PRR)

PRR are conserved among species from plants and fruit flies to mammals. PRR recognize a plethora of conserved molecular structures usually termed PAMPs which are present on a large group of microbes (including pathogens and commensals) [7]. These conserved structures are essential for the survival of the microorganism and in addition, PAMPs are not expressed by the host. PRR are usually constitutively expressed in the host, detect the pathogens regardless of their life cycle, and are present in serum, intracellularly, extracellularly and on the cell surface. When pathogens are present in blood, serum PRR opsonize them, leading to enhanced clearance by phagocytic action of neutrophils and macrophages [8]. TLRs, one of the most studied families of PRR, upon stimulation, initiate common nuclear factor kappa B (NF-κB)/activator protein-1(AP-1) and distinct interferon regulatory factor (IRF) pathways [9]. These in turn lead to transcription of immune response genes that coordinate innate immunity and initiate adaptive immunity against pathogens. Recently, TLR-independent pathways have been identified. The cytoplasmic caspase-recruiting domain (CARD) helicase, such as RIG-1/melanoma differentiation-associated gene 5 (MDA5) initiates antiviral immunity by stimulating the production of interferons (type I) via adaptor interferon-β promoter stimulator 1 (IPS-1) [10, 11]. Also, NLR activate NFκB or inflammasomes when induced by bacterial molecules [12]. An important mannose receptor, Dectin-1, is involved in detection of antifungal antigens and promotes phagocytosis and activates NFκB [13]. In vivo, upon encounter with a pathogen, the IIS will recognize various PAMPs associated with the particular organism. Different signaling pathways are activated, some of which are dependent on the adaptor molecule myeloid differentiation 88 (MyD88). Interestingly enough, most of the PRR receptor signaling pathways activate the NFκB pathway.

1.1.2 TLR

Toll was first identified as a developmental gene product essential for the development of embryonic dorsoventral polarity in Drosophila [14]. Later, it was observed that Toll plays a critical role in the antifungal response of these flies [15]. Subsequently, other homologous receptors were found in vertebrates and denoted as TLRs [16, 17]. The TLRs family currently consists of 13 known members in mammals which recognize a whole array of PAMPs [18]. TLR are type I transmembrane glycoproteins and consist of an extracellular domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling, Toll/IL-1R (TIR) interacting domain [17]. These receptors are found both intracellularly and on the cell surface. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface while TLR 3, 7, 8 and 9 are found exclusively in intracellular compartments such as endosomes [19]. The TLR family can be further divided into subfamilies based on the primary sequences they recognize, for example TLR1, 2 and 6 recognize lipids whereas TLR 3, 7, 8, and 9 recognize nucleic acids (Table 1.1). However TLR4 can recognize a very divergent collection of ligands such as lipopolysaccharide (LPS), the plant diterpene paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibronectin and heat shock proteins [20-23]. TLR are expressed on immune such as Mϕ, DC, B cells, specific types of T cells, and non-immune cells such as fibroblasts and epithelial cells. Although the expression of TLR is constitutive, they can also be modulated rapidly during infection and are differentially expressed on different subsets of cells [7]. Another important aspect is the distribution of the PRR, such as TLR, on immune system cells. All TLR are not distributed equally on all cell types suggesting that particular cell types are more specialized to recognize particular PAMPs and deal with the subsequent threat.

For example, human myeloid DC (mDC) express TLR1, 2, 4, 6, but not TLR7 and 9, whereas plasmacytoid DC (pDC) express only TLR7 and 9 [24, 25]. Upon engagement of these distinct subsets of DCs by TLR agonists, a different innate immune profile is obtained. pDC are known for their potent type I IFN secretion following stimulation with TLR9 agonist whereas myeloid DC produce limited amounts of IFN [26]. Upon engagement of any of the TLR, the TIR domain recruits adaptor

molecules leading to the activation NFκB/AP-1 and IRF pathways. There are five main adaptor molecules in the TLR signaling pathway that have been identified as crucial. These are myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR domain-containing adaptor protein-inducing IFNβ (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2) and sterile α- and armadillo motif-containing protein (SARM) [27].

Table 1.1: TLR members each recognizing unique features (PAMPs) from pathogens and are expressed on some major cell types.

TLR	Agonist	Organism	Major Cell type	Reference
1	Triacyl lipopeptides	Mycobacteria	Mϕ, cDC, neutrophils, mast cells	[28]
2	LTA, Zymosan, PamCSK4, MALP2, lipoarabinomanna	G^+ve bacteria, yeast, mycobacteria	Mϕ, cDC, neutrophils, mast cells	[29]
3	dsRNA, poly(I:C)	Virus	cDC, Mϕ (mouse), endo/epithelial cells	[30]
4	LPS, mannan, GIPLs, envelope proteins, Heat shock proteins	G^-ve bacteria, Candida albicans, Trypanosoma, RSV and MMTV	Mϕ, cDC, neutrophils, mast cells, eosinophils	[31]
5	Flagellin	Bacteria	Monocytes, cDC, iEC	[32]
6	Diacyl lipopetides, LTA, Zymosan	Mycoplasma, G^+ve bacteria, yeast	Monocytes, mast cells, cDC, neutrophils	[33]
7	ssRNA, Imiquimod,Resiquimod (R848), synthetic polyU RNA, siRNAs	Virus	pDC, neutrophils, eosinophils	[34]
8	Resiquimod (R848), ssRNA	Virus	Monocytes, cDC, mast cells, neutrophils	[35]
9	CpG DNA, hemozoin	Bacteria, plasmodium	pDC, B cells, NK cells, eosinophils, neutrophils	[36]
10	Not known		pDC, B cells	
11	Profillin-like molecule	Toxoplasma gondii, uropathogenic bacteria	Mϕ, epithelial cells	[37]
12	Not known			
13	Not Known			

Mϕ -Macrophages: pDC-plasmacytoid dendritic cells: cDC-conventional DC: IEC-intestinal epithelial cells: NK-Natural Killer cells.

The first four adaptor molecules have been shown to be involved in TLR signaling pathways. The last one is a negative regulator of the pathway. The TLR signaling can be sub-divided into 2 pathways: MyD88-dependent and MyD88-independent pathways. All known TLRs activate MyD88-dependent pathways except for TLR3. In addition, TLR4 signals through both MyD88-dependent and independent pathways [38]. All TLR pathways lead to the activation of NFκB, mitogen-activated protein kinase (MAPK), AP-1 and IRF pathway. TIR domain of the TLR receptor directly recruits MyD88. First, this complex recruits IL-1R-associated kinase 4 (IRAK4) and then IRAK1, and TRAF6 (tumour necrosis factor receptor-associated factor 6) interacts with the TLR-MyD88-IRAK4 complex. Subsequently TAK1 (transforming growth factor β-activated kinase) is actively recruited by the five adaptor complex. The ubiquitinylating factors UEV1A (ubiquitin-conjugating enzyme E2 variant1) and UBC13 (ubiquitin-conjugating enzyme 13) modify and activate TRAF6 and TAK1 on the complex. This leads to the activation of the inhibitor of NFκB kinase (IKK) complex and NFκB, and the upstream kinases for p38 and JUN N-terminal kinase (JNK) (Fig. 1.1) [39]. MyD88 is an important molecule and is subjected to negative regulation because TLR need to be tightly controlled as they activate potent immunostimulatory responses [27].

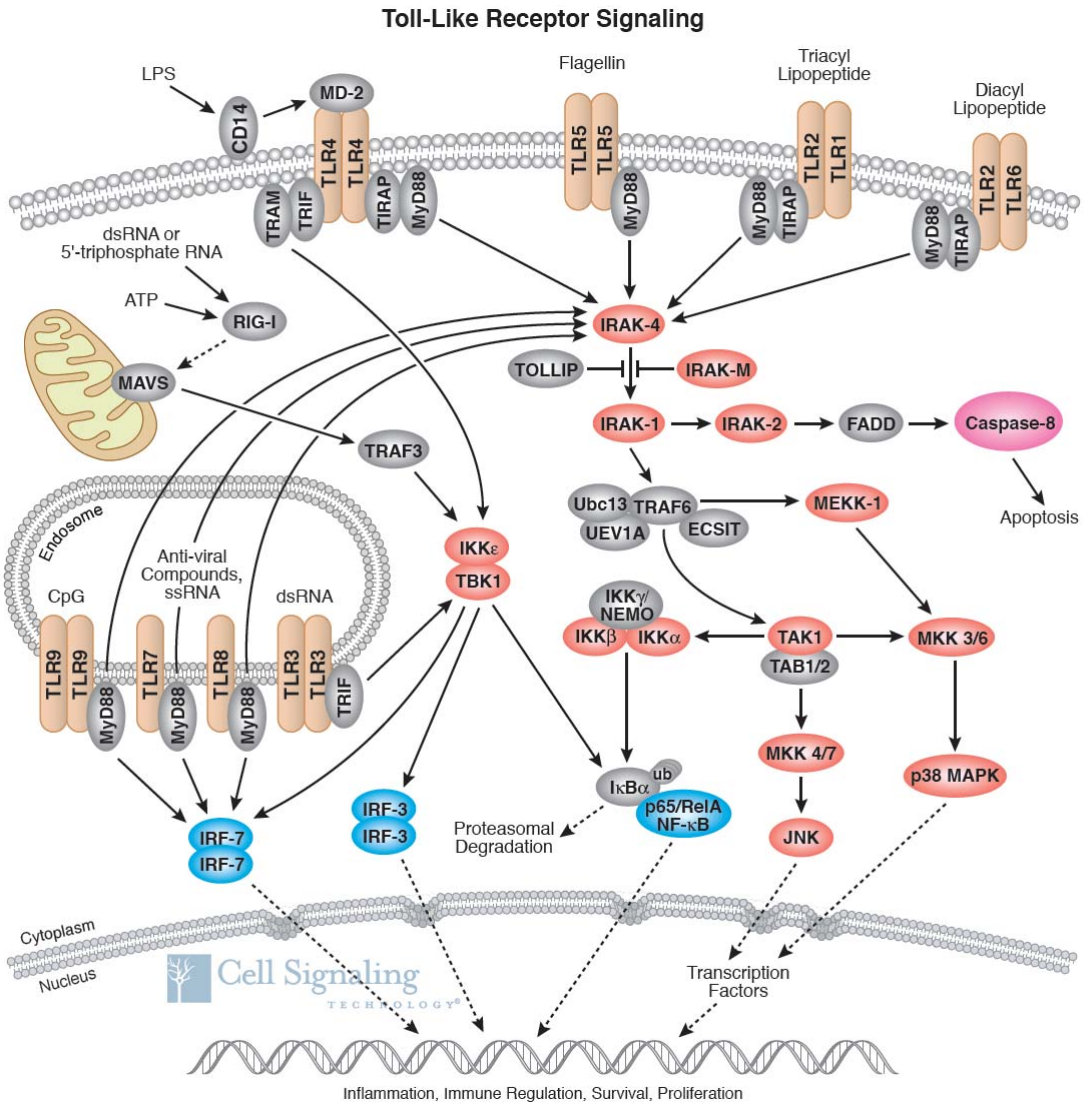

Fig. 1.1: General TLR pathway showing various adaptor molecules with negative regulators (adapted from Cell Signaling Technology (www.cellsignal.com).

One of the negative regulators of MyD88 termed MyD88s interferes with IRAK4 recruitment and FAS-associated via death domain (FADD). MyD88s also couples to IRF5 and IRF1 [39], and thus interfere with the induction of IFN transcription. Interleukin-1 receptor-associated kinase M (IRAK-M) also inhibits signaling by the release of IRAK1 and IRAK4 from MyD88. Both TLR2 and TLR4 signaling involve a bridging adaptor molecule, MAL, which is needed for the

recruitment of MyD88. BTK (Bruton's tyrosine Kinase) and SOCS1 (Suppressor of cytokine signaling 1) promote MAL degradation and thereby regulate the recruitment of MyD88. TLR7, 8, and 9 activate the MyD88-IRAK4 pathway and through TRAF6 and TRAF3 also lead to the activation of IRF7. Mice deficient in MyD88 show profound unresponsiveness to ligands for TLR 2, 5, 7 and 9 [38], while mice deficient in TRIF show unresponsiveness to agonists for TLR3 [40].

1.1.3 TLR9 and CpG motif recognition

Bacterial and viral DNA is the natural ligand for TLR9 but synthetic CpG oligodeoxynucleotides (ODN) are commonly used as TLR agonists because they were shown to be present in higher frequency in bacterial and viral genomes. CpG ODN bind to TLR9 [41], leading to stimulation of innate and adaptive immune responses. The distinct compartmentalization of the TLR9 receptor is believed to be an important factor in its ability to trigger specific responses to a variety of harmful intra- and extracellular microorganisms [42]. An important aspect of TLR9 recognition is the discrimination between self and foreign DNA, which is achieved through structural differences between vertebrate and microbial DNA. Bacterial and viral DNA has a frequency (1/16 instead of 1/64) of unmethylated CpG dinucleotides compared to vertebrate DNA [43]. Therefore, it is thought that the vertebrate IIS has evolved to detect unmethylated CpG dinucleotides as a way of sensing the presence of infection. However, in certain pathogenic situations, such as in systemic lupus erythematosis, TLR9 can recognize mammalian DNA within immune complexes, leading to autoimmunity [44]. Recent reports suggest that most of the observed immune effects of CpG ODN are an artifact of modification of the ODN backbone [45] but this remains unresolved.

In this regard, normal phosphodiester (PO) CpG ODN are rapidly degraded inside cells such that their ability to stimulate CpG-induced responses is reduced. Thus the synthetic ODN backbone has been modified to a phosphorothioate (PS) and this provides a high degree of nuclease resistance, greatly stabilizing the ODN against

degradation [46]. In addition, the PS ODN generally binds more avidly to cell membranes leading to a higher cellular uptake [47].

There are three known classes of CpG ODN which are classified according to unique structural and biological characteristics. A-class CpG ODN contains poly G motifs with PS linkages at the 5' and 3' ends and a PO palindromic CpG-containing sequence in the ODN center [24]. A-class CpG ODN has the ability to strongly activate plasmacytoid DC to secrete IFNα and is also effective in activating natural killer (NK) cells [48, 49]. However A-class is a poor inducer of B cell proliferation. In contrast, B-class CpG ODN has a fully PS-modified backbone with one or more CpG motifs but no poly G motif [49]. They induce strong B cells proliferation and pDC maturation but are poor inducers of IFNα secretion in pDC. The biological characteristics of C-class CpG ODN are intermediate between A- and B-classes; these ODNs induce both B cell-activation and IFNα secretion [50, 51]. This classification is largely based on effects of various CpG ODN on human PBMC. However, it has become evident that the classification may not hold true in immune cells from other tissues, such as lymph nodes (LN) (See chapter 3).

The immune stimulatory effects of an ODN depend mainly on the precise bases flanking the CpG dinucleotide. It was observed that the optimal CpG motifs for different species vary according to the flanking bases. The best motifs for activating mouse or rabbit immune system cells have the general formula, purine-purine-CG-pyrimidine-pyrimidine, GACGTT [52-54]. For human cells, the optimal motif is GTCGTT [55]. This latter motif has also been found to be optimal for many other vertebrates including sheep, cows, cats, dogs, goats, horses, pigs and chickens [53, 56].

1.2 Innate Immune responses induced by CpG ODN

TLR9 activation with CpG ODN results in induction of the IIS and shapes the adaptive immune responses. In general the innate immune responses induced by CpG ODN involve the secretion of IFNα, IFN-inducible chemokines and cytokines, pro-inflammatory cytokines such as IL-6, TNFα, IL−12 and anti-inflammatory cytokines

such as IL-10 and IL-1RA. CpG ODN also induces NK cell lytic activity, monocyte expression of TNF-related apoptosis-inducing ligand (TRAIL) [57] and neutrophil activation, migration and bacterial uptake [58-60]. On the adaptive end, CpG ODN induces B cells to undergo isotype switch and secrete antibody, but also IL-6, IL-10 and IL-12. CpG ODN indirectly induces Th1 cells to differentiate. A few studies have found that T cells highly express TLR after activation via the T cell receptor (TCR). These activated T cells respond to TLR agonists which can then modulate T cell function [61, 62]. Responses to CpG ODN have been found to vary according to the range of cells expressing the TLR9 receptor [43]. In mice, most cell types, including B cells, pDC, cDC, monocytes, NK cells, T cells, Mϕ, and neutrophils, express TLR9 and therefore CpG ODN is highly potent in mice [9].

1.2.1 Dendritic cell responses to CpG ODN

There are various distinct subsets of murine and human DC and they express different subsets of TLR which allow them to modulate different patterns of immune responses to different pathogens. In humans, one of the DC subsets that expresses high level of TLR9 is pDC ($B220^+$, $Ly6C^+$) while myeloid $CD11c^+$ pre-DC do not express TLR9, but do express TLR4. Thus, in humans, CpG ODN directly activate pDC to secrete cytokines [IFNα, TNFα, IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF)], and chemokines (IL-8, IP-10), and to increase surface expression of major histocompatibility complex (MHC) class II, inter-cellular adhesion molecule 1 (ICAM-1) and co-stimulatory molecules (CD40, CD54, CD80 and CD86) [25, 26, 63, 64]. pDC also mature and become $CD83^{bright}$ leading to increased activation of allogeneic T cells [65] and resistance to IL-4-induced apoptosis. pDC are known for their role as the primary source of IFNα, which is rapidly produced during CpG stimulation. The functional properties of DC vary according to their location. Murine Peyer's patch DC produce higher levels of IL-10 and lower IFNα than spleen DC upon stimulation with CD40L or microbial stimuli [66]. Further PP DC, but not spleen DC, can induce the differentiation of naïve T cells into T regulatory cells upon stimulation with CpG ODN [67]. Thus the anatomical location of cells plays a role in their

responses to stimulation. However, the local factors in the PP that modulate the behavior of pDC are not known.

1.2.2 B cell responses to CpG ODN

B cells are the other main cell type that express TLR9 in humans and hence can be stimulated directly by CpG ODN. Upon stimulation with CpG ODN, B cells enter the G1 phase of the cell cycle and secrete IL-6 within hours, a requirement for B cell secretion of IgM [68]. IL-10 is produced by B cells as a counter-regulatory mechanism to reduce the effects of proinflammatory cytokines such as IL-12 [69]. Further, CpG ODN induce B cells to increase their expression of the Fcγ receptor and costimulatory molecules (MHC II, CD80 and CD86) [54, 70, 71] and, in humans, CD40 and CD54 are also upregulated [55]. It has been reported that low concentrations of CpG ODN strongly synergize with B cell receptor (BCR) signals leading to increased B cell proliferation and antigen-specific antibody and IL-6 secretion [54]. CpG ODN has also been reported to have anti-apoptotic activity on isolated primary B cells [52] and to protect B cells against Fas-mediated apoptosis [72]. Moreover, TLR9 mRNA expression has been found to vary in B cell subtypes. In humans, it has been reported that only activated and memory B cells express high levels of TLR9 and therefore respond directly to CpG ODN [73, 74]. However, recent evidence suggests that naïve human B cells also respond directly to CpG ODN [75]. However, these studies were done using peripheral blood B cells and whether B cells from other immune tissues respond similarly has not been explored in details. The few studies that have addressed this issue found that all subsets of B cell populations in tonsils respond directly to TLR stimulation, including CpG ODN [76, 77]. B cells from tonsils upregulate CD80, CD86, CD40, CD23 and MHC class II following stimulation by CpG ODN. However, their proliferative responses, and antibody (IgM and IgG) and cytokine (IL-6 and IL-10) secretion were not significantly induced by CpG ODN. These tonsillar B cells required costimulation with CD40L and CpG ODN for significant proliferation and cytokine and antibody secretion [78]. Again, these results suggest that the anatomical microenvironment can influence the profile of CpG ODN responses.

1.2.3 Immune cells stimulated by CpG ODN

Other immune cells such as macrophages (Mϕ) and NK cells have been reported to be directly or indirectly activated by CpG ODN. In humans, Mϕ and natural killer cells do not constitutively express TLR9 but can modulate its expression [25, 79]. In mice, CpG induces the secretion of cytokines such as TNFα by TLR9-expressing Mϕ [80]. However, expression of inducible nitric oxide synthase and production of nitric oxide in Mϕ is not due to direct CpG activation but rather requires IFNγ priming [81]. Initially, upon CpG stimulation, Mϕ increase their ability for antigen processing and presentation but after 18 hours, these processes are downregulated [82, 83]. It has been shown that purified monocytes do not express TLR9 and thus are not activated directly by CpG ODN in humans [25]. Recently it has been proposed that while monocytes are not directly activated by CpG ODN in bovine species, they are required for B cells proliferative responses to CpG ODN (Arsenault et al., 2009, in press).

Murine NK cells are not directly activated by CpG ODN but are affected by cytokines such as type I interferon secreted as a consequence of CpG ODN stimulation. Thus CpG ODN seems to perform as a costimulatory signal for murine NK cells. In humans, ODN containing CpG palindromes reportedly can activate human NK cells directly [84]. However, this report did not use highly purified NK cells and it may be that the NK cells could have been activated indirectly via contaminating cells.

Similar to NK cells, there are conflicting reports on whether T cells are directly activated by CpG ODN. It has been shown that human peripheral blood T cells expressed TLR 1, 2, 3, 4, 5, 7 and 9 mRNA to varing degrees [85-87]. It seems that purified T cells in humans can be induced to proliferate by CpG ODN as long as the TCR is strongly cross-linked [84]. Selective expression of TLR appears to occur on specific subsets of T cells. Human regulatory T cells (T_{regs}) ($CD4^+$ $CD25^+Foxp3^+$) were found to express TLR5 and 8, whereas $CD25^-$ $CD4^+$ T cells do not express these two TLR [88]. Stimulation of T_{regs} with TLR5 agonist, enhances the suppressive capacity and induces higher expression of forkhead box P3 (Foxp3) [89] whereas stimulation with TLR8 reverses the suppressive nature of T_{regs} [90]. There are only a few reports on the effect of CpG on neutrophils and it appears that CpG ODN does not directly activate

these cells but it may improve their ability to phagocytose and increase their production of reactive oxygen species [91]. Thus, in humans and possibly in ruminants, the main cells that are activated directly by CpG are B cells and pDC [92, 93].

1.3 Simultaneous multiple TLR stimulation

1.3.1 Multiple TLR stimulation

Most studies on TLR activation have explored the effects of exposure to a single TLR agonist. However, during the course of an infection, the host immune system is usually presented with multiple TLR and non-TLR agonists and therefore the responses observed are a net effect of these signals, which are possibly synergistic or inhibitory to each other. For this reason, recent studies have explored multiple TLR stimulation, which can profoundly alter the effects of each other. Therefore the order in which the cells are exposed to TLR agonists may have a significant impact on the ensuing immune responses. For example, Napolitani and colleagues reported that in mice and human DC, TLR3 and 4 synergize with TLR7, 8 and 9 stimulation [94] to increase levels of IL-12, and IL-23 and the Delta-4/Jagged ratio, which enhanced and sustained T helper 1 (Th1) polarization. It is known that all TLRs, except for TLR3, use the MyD88-dependent pathway and that TLR4 uses both pathways. Recently, Bagchi and colleagues showed that a combination of TLR agonists that employed MyD88-dependent and -independent pathways have synergistic outcomes as indicated by increased cytokine levels and NF-κB translocation in both *in vivo* and *in vitro* experiments [95]. They also showed that when used simultaneously or sequentially, all MyD88-dependent TLR agonists are inhibitory to one another. Thus they concluded that simultaneous and sequential activation of both MyD88-dependent and MyD88-independent pathways resulted in synergistic TLR responses, whereas activation with multiple agonists using the same pathway resulted in inhibition [95].

TLR agonists have been shown to induce the secretion of both inflammatory (TNFα, IL-12) and anti-inflammatory cytokines (IL-10). In one recent study, Hirata and colleagues showed that TLR2 agonist (Pam3CSK4) alone does not induce the secretion

of IL-10 and that TLR4 agonist (ultra pure LPS) alone induces the secretion of proinflammatory cytokines such as IL-12 and TNFα in conventional murine DC. Co-stimulation with both TLR2 and TLR4 agonists lead to high level of IL-10 but not of IL-12 and TNFα [96]. Therefore, TLRs can also cross-regulate their effects, resulting in either an inflammatory or anti-inflammatory state.

1.4 Overview of the Mucosal Immune system

The mucosal immune system is at the interface between the internal and external environment and interacts with air, water, microbes and food antigens. The intestine, respiratory and urogenital mucosal tissues have a surface area that is larger than the skin by 200-fold. The mucosal tissues constitute efficient barriers, preventing pathogens from gaining access to the host milieu but they are also a major route of invasion by pathogenic microorganisms [97]. The microenviroment of the immune tissues at these mucosal barriers dictate the ensuing immune responses.

The mucosal surfaces such as intestine are constantly exposed to food antigens and harmless commensal bacteria as well as many dangerous pathogens. Strong immune responses are needed to protect this tissue from pathogens. However immune response against food antigens and non-pathogenic organisms would not only be wasteful but would also lead to constant inflammation and pathology. This type of inflammatory disorder has been found in humans such as coeliac disease, which is a chronic inflammation of the upper small intestine in humans caused by immunological hypersensitivity to the α-gliadin component of wheat gluten [98]. Thus, active regulatory mechanisms are required to prevent responses to such harmless microbial molecules and dietary antigens both at the systemic and mucosal levels [99]. The mucosal tissues have special features, including unique ontogeny, specialized cells and organs (M cell and Peyer's patches) for antigen uptake, unique subsets of antigen-presenting cells (APC), unique B and T cell populations, and high level production of non-pathogenic immunoglobulin A (IgA) antibodies [100]. Moreover the levels of mucus and antimicrobial peptides secreted not only protect the epithelial cells but

influence colonization by microorganisms [101]. More recently, the involvement of TLR receptors has been implicated in gut homeostasis [102, 103]. Thus the mucosal immune system must have the ability to discriminate between pathogens and commensals and food antigens.

1.4.1 Gut-associated lymphoid tissues

In humans, Peyer's patches (PP), small intestinal isolated lymphoid follicles, appendix and colonic lymphoid follicles constitute the gut-associated lymphoid tissues (GALT). Although many studies have been performed in rodents, there are large phenotypic and structural differences in the GALT of mice, humans and other species such as sheep. These differences are: (i) mice do not have fully developed PP at birth while, in humans and sheep, PP are fully developed and functional at birth; (ii) the number of PP in humans and sheep are higher than in mice; (iii) there are more cryptopatches in mice than in human and sheep; and (iv) finally, there are more γδ T cells in the gut epithelium of mice than in humans [100]. These differences suggest that the functions of the GALT between humans and mice would be different. GALT can be divided into (i) inductive sites consisting of Peyer's patches and mesenteric lymph nodes (mLN) and (ii) effector sites which are a network of immune cells scattered throughout the epithelium and lamina propria of the intestine.

1.4.2 Peyer's patches

PP named after the Swiss physician Johann Conrad Peyer, are secondary lymphoid tissues containing germinal centers, similar to lymph nodes. PP develop in specific regions of the gut during fetal development and some persist in adult [104]. PP consist of a collection of large B cell follicles surrounded by T cell areas. The follicle-associated epithelium (FAE) is comprised of the single columnar epithelial cells that separates the lymphoid tissues from the gut lumen whereas the subepithelial dome (SED) is the diffuse area just below the epithelium (Fig. 1.2) [105]. FAE differs from

other epithelia because it is infiltrated by large numbers of B cells, T cells, Mϕ and DC and expresses lower levels of digestive enzymes [106]. An important feature of the FAE is the presence of microfold (M) cells that are specialized enterocytes which lack surface microvilli and the normal over-layer of mucus. The primary function of M cells is to sample antigens from the intestinal lumen (reviewed by Corr *et al* [107]). However, they can also bind invasive pathogens such as *Salmonella*, *Shigella*, *Yersinia* and reoviruses and particulate antigens [108]. B cells that are present near the epithelial cells secrete Notch 1 and LTα1β2, which regulate epithelial cell differentiation into M cells [109].

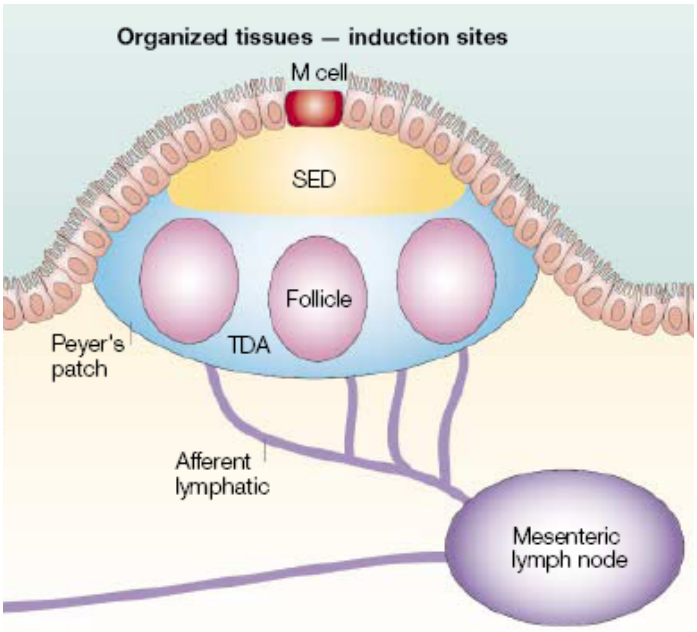

Fig. 1.2: Diagramatic representation of Peyer's patch (Inductive site) showing M cell and the follicular region (adapted from Mowat, 2003).

1.4.3 Distribution and function of PP in sheep

PP can be found in two distinct locations in the small intestine, namely the ileum and jejunum. Jejunal and ileal PP have distinct developmental, structural and functional attributes as shown in Table 2.1. The JPP is an efficient site for the induction of both mucosal and systemic immunity [110], whereas the IPP is a major source of cells for the total B cell pool and appears to play a role in the antigen-independent diversification

of the immunoglobulin repertoire [111]. Therefore the JPP but not the IPP is an immune induction site in the intestine.

Table 2.1: Differences between sheep IPP and JPP (Adapted from [100])

IPP	JPP
Continuous single patch	Discrete patches (25-40 patches)
100,000 follicles	10,000 follicles
Present at birth, involutes 6-15 months	Present at birth and throughout life
70% of total B cell pool	5% of total B cell pool
95% B cells	35-45% B cells
Less than 0.5% T cells in follicle	10-15% T cells in follicle
2-3% $CD5^+$ B cells	30-40% $CD5^+$ B cells
No M cells	Contains M cells
Generation of primary antibody repertoire	Induction of mucosal Immunity

1.4.4 Sheep PP B cells

Two phenotypic distinct populations of B cells have been reported in sheep [112-115]. One subpopulation expresses $CD11b^+$, $sIgM^{hi}$, and $CD11c^+$ but not CD21 or L-selectin. These B cells populate the splenic marginal zone but are absent from splenic and ileal PP follicles. They do not recirculate but remain in blood and spleen. The second population of B cells are $CD21^+$, L-$selectin^+$ and IgM^{lo}. These cells populate the PP and splenic follicles but are absent from the splenic marginal zone. They are the only B cells in afferent lymph, efferent lymph and all lymph nodes. $CD21^+$ B cells are recirculating cells as they can be detected in efferent lymph by 16 hrs after cannulation and peaking at 24-30 hrs when using a fluorescent trace on that population.

1.4.5 Mucosal DC

DC are professional APC that take up and process antigen for presentation to naïve T cells in lymph nodes. There is an extensive network of dendritic cells present in the GALT, including conventional DC ($CD11c^{hi}$) and pDC. They are derived from hematopoetic cells that seed most tissues and thus DC control tolerance and immune response at those locations. Mucosal DC have been shown to have distinct functional properties. For example murine PP DC secrete higher levels of IL-10 than spleen DC in response to TLR stimulation [116]. These differences suggest that DCs at the mucosal sites are influenced by various local environment factors that affect their ability to respond. DC can be classified according to their cell surface receptor expression. In murine PP and mLN, there are three subsets of so-called conventional DC namely $CD11c^{hi}CD11b^{+}CD8\alpha^{-}$, $CD11c^{hi}CD11b^{-}CD8\ \alpha^{+}$ and $CD11c^{hi}CD11b^{-}CD8\alpha^{-}$ cells [66, 117]. Murine PP $CD11c^{hi}CD11b^{-}CD8\alpha^{+}$ and $CD11c^{hi}CD11b^{-}CD8\ \alpha^{-}$ DC have been shown to secrete IL-12 and subsequently induce IFNγ in T cells [66], but murine PP $CD11c^{hi}CD11b^{+}CD8\alpha^{-}$ DC produce IL-10 and prime Th2 cells. The expression of chemokine receptors have also been used to categorize DC. In the FAE of mice, $CX3CR1^{+}$ DC were closely associated with the epithelial cell in the steady state while $CX3CR1^{-}$ DC were found to be mostly in the lamina propria [118]. The pDC subset has a surface marker $CD11c^{mid}$ and is present in both murine PP and mLN, but has not been found in the lymph [67, 119, 120].

In sheep, the panel of antibodies that has been used to stain and identify DC in afferent lymph include CD1, mannose receptor, CD11c and CD21. Sheep DC have been described as $CD1^{+}$, $CD11c^{+}$,and $CD11b^{+}$, but $CD21^{-}$ [121]. Ovine prefemoral afferent lymph DC were found to express high levels of MHC I and II molecules, CD1 and CD58 (an adhesion molecule) [122]. In vitro-generated ovine DC were found to weakly express CD11c and mannose receptor [123]. Moreover DC obtained by cannulation of sheep were characterized and found to crossreact with human DC marker (CD83 and CMRF-56) monoclonal antibodies [124].

1.4.6 Mechanisms leading to intestinal homeostasis

The intestine is always exposed to PAMPs from commensal bacteria and food antigens but seldom mounts an inflammatory response to such molecules and antigens. However, the mucosal immune system can mount strong immune responses to pathogens. Therefore there must be mechanisms by which the mucosal immune system dampens responses to commensals and food antigens and thereby maintains intestinal homeostasis. TLR detect conserved microbial molecules that trigger innate and adaptive immune responses and have been reported to be expressed in a variety of cells in the intestine, including intestinal epithelial cells (IEC), Mϕ, DC and B cells. It is thought that TLR signaling must be tightly regulated in the intestine to prevent unwanted or prolonged inflammation. How TLR responses are regulated in the intestine is not fully understood, but several mechanisms have been proposed.

1.4.6.1 Downregulation of TLR and their adaptors on immune system cells

The lamina propria and PP are rich with immune system cells such as DC, Mϕ, B cells and T cells that express TLRs. One of the proposed mechanisms regulating TLR responses in intestine is downregulation of the expression of TLRs and their adaptors on immune cells. For example, lamina propria Mϕ have low expression of TLR 2 and 4 in uninflamed intestinal tissue [125]. Furthermore intestinal Mϕ lack both CD14 and CD89 [126]. CD14 is a surface cell receptor that is required in TLR4 stimulation by LPS. Moreover, Smythies and colleagues showed that intestinal Mϕ do not express a number of innate immune receptors such as CD14 (LPS), CD89 (Fcα), Fcγ (CD64, CD32, CD16), CR3, CR4, and receptors for growth factors (IL-2, IL-3) [127]. Interestingly, these cells have acquired profound inflammatory anergy but they retain their full phagocytic and bactericidal activity. Therefore the absence or low expression of TLRs and other innate immune receptors may contribute to low levels of inflammation in normal intestinal tissues.

1.4.6.2 Inhibitors in TLR signaling pathways

Negative regulation of TLR signaling is one of the other mechanisms by which the intestine can maintain homeostasis. It has been suggested that initiation of TLR signaling is tightly regulated, as prolonged activation can lead to uncontrolled inflammation. Various mechanisms appear to control TLR activation in intestinal epithelial cells including several negative regulators, such as IRAK-M, Toll-interacting protein (TOLLIP), single immunoglobulin IL-1R-related molecule (SIGIRR), A20 (zinc finger protein with ubiquitin-modifying activity), NOD2, peroxisome proliferator-activated receptor gamma (PPARγ) and suppressor of cytokine signaling (SOCS) [128]. TOLLIP has been shown to inhibit the TLR pathway by suppressing IRAK molecules and is upregulated in response to non-pathogenic bacteria [129, 130]. PPAR-γ is a member of a nuclear receptor family. It is involved in the repression of a distinct subset of TLR [131]. Kelly and colleagues demonstrated that PPAR-γ associates directly with the reticuloendotheliosis viral oncogene homolog A (relA) subunit of NFκB and limits the translocation of NFκB to the nucleus [132]. The other inhibitors also affect the association of adaptor molecules in the pathway. For example IRAK-M blocks the formation of IRAK1-TRAF6 complexes and prevents the dissociation of IRAK1-IRAK4 from the TLR receptor complex, thereby inhibiting downstream activation of NFκB. Therefore negative regulators could play a role in maintaining gut homeostasis.

1.4.6.3 Cellular localization of TLR9

Another mechanism for intestinal homeostasis that has been recently proposed involves the expression of bacteria DNA receptor TLR9, in intestinal epithelial cells (IEC). TLR9 is usually found in the endosomal compartment in immune cells. It has been recently reported that cell surface TLR9 has been observed in intestinal epithelial cells, paneth cells and tonsillar epithelial cells [133]. This is a peculiar location, as TLR9 could be readily engaged by commensal bacterial or viral DNA in the intestinal lumen and induces pro-inflammatory responses. However Lee and colleagues have

proposed a novel TLR9 signaling mechanism that may explain how IEC avoid inflammation in a microbe-rich environment under steady state [103, 134]. An important feature of IEC is the polarity of these cells. On one side, the apical surface faces the lumen which contains abundant antigens, while the basolateral side faces the lamina propria which contains vast amount of immune cells. Lee and colleagues investigated whether this polarity affects the immune response to TLR ligands [103, 134]. They showed that while the expression of TLR9 was on both apical and basolateral surfaces of IEC, only the latter responded to CpG ODN by activating NFκB. The authors infer that the differential response to CpG ODN was the result of different signaling pathways in response to apical and basolateral stimulation. They demonstrated that p105 (a precursor of NFκB) and IkBα (inhibitor of NFκB) degrade only during basolateral stimulation but not upon apical stimulation of TLR9. They further found that after apical stimulation, subsequent TLR re-stimulation whether it is apically or basolaterally, resulted in unresponsiveness. Therefore, the authors proposed that apical stimulation of TLR9 could induce a state of unresponsiveness to proinflammatory TLR signaling from both apical and basolateral IEC surfaces. This may be due to an increased threshold for NFκB activation.

1.4.6.4 Th2 cytokine bias

One of the special features of the intestine is that it is a Th2-biased microenvironment and thus it is usually under the influence of Th2 cytokines such as IL-4, IL-5, and IL-13. This skewed cytokine profile in the intestine is thought to influence the TLR expression profile. In 2006, Mueller and colleagues proposed that the Th2 cytokine environment of the gut plays an important role in balancing TLR signalling in human IEC. They showed that Th2 cytokines downregulate TLR expression and function (IL-8 secretion) in IEC, thereby preventing the host from mounting excessive responses to commensals [135]. The authors proposed that this may be an important mechanism by which the gut maintains homeostasis.

1.4.6.5 Immunoregulatory cytokines (IL-10, TGFβ)

Interleukin-10 is a critical immunoregulatory cytokine and is produced mainly by DC, Mϕ, B cells and T_{regs}. IL-10 has pleiotropic effects on various hemopoeitic cells. IL-10 has been reported as a stimulator of NK cells and B cells in mice and humans [136], but it was first shown to have the ability to suppress activation and effector function of T cells, monocytes and Mϕ [137]. The role of IL-10 is to limit and terminate inflammatory responses. For example, IL-10 inhibits the production of IL-12, IL-18 and MHC II upregulation by APC [138]. This anti-inflammatory role is demonstrated in IL-10-deficient mice, which spontaneously develop colitis [139]. This observation suggests an important role for IL-10 in the maintenance of intestinal homeostasis. IL-10 also plays a key role in autoimmune diseases, as shown in disease models such as experimental autoimmune encephalomyelitis (EAE) where it can downregulate the active inflammatory responses [140]. We have shown that IL-10 is secreted by PP $CD21^+$ B cells *in vitro* and that it has suppressive effects on other immune cells, as indicated by downregulation of CpG-induced IFNα, IFNγ and IL-12 in the intestine. TGFβ has also been suggested to downregulate responses in the intestine, thereby contributing to gut homeostasis. It has been shown that TGFβ is constitutively expressed in the intestine [141] and contributes to PP B cells switching to IgA [142]. Moreover, TGFβ suppresses IFN responses by pDC, but maintains lower level of IL-12 secretion [143]. Regulatory T and B cells (T_{regs} and B_{regs}) present in the gut are thought to be the major sources of IL-10 and TGFβ, and therefore to play a major role in preventing potentially tissue-damaging T cell responses.

1.5 B lymphocytes

1.5.1 TLR9 in B cell activation

Krieg and colleagues reported that unmethylated CpG dinucleotides induced murine B cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo* [54]. This was the first demonstration that bacterial or synthetic DNA triggers direct activation of

B cells. Thus they showed that purified B cells can be activated with microbial PAMPs. However, the B cells were purified by Krieg and colleagues on the basis of MHC class II expression, which suggests that other leucocytes such as Mϕ and DC could have been present and thus could have confounded their results. Moreover, when TLR9 ligand was used alone, the level of proliferation was low (less that 2500 cpm) but on activation through BCR, the level of proliferation was high (above 15000 cpm). This suggests that BCR signaling is required for significant activation of B cells through TLR9. This is similar to the two signal model where signal 1 (BCR signal) and 2 (T cell signal) are needed for B cell activation. However signal 2 originates from innate immune receptors as proposed by Janeway [2]. Subsequently, it was shown by Leadbetter and colleagues [144], that DNA-containing chromatin-IgG complexes have the ability to activate autoreactive B cells to produce antibody against self IgG (rheumatoid factors) by engaging BCR and TLR9 in sequential fashion. Thus the dual BCR-TLR stimulation is well established [145-147]. Moreover, the induction of antigen-specific T-dependent B cells responses has been shown to also require TLR activation of B cells for optimal antibody production in mice and humans [73, 148]. More recently, a study was performed where subsets of murine naive B cells, namely B-1a, B-1b, marginal zone (MZ) and follicular B cells (FO) were stimulated with TLR agonists [149]. They showed that upon TLR stimulation, B-1 and MZ B cells differentiated primarily into mature plasma cells but not follicular B cells. Thus TLR9 may be required for T-dependent B cell responses, which adds another layer of complexity to the system in terms of 'signal 3'. Therefore the model for B cell activation has been suggested to consist of three signals, where signal 1 (BCR signal), signal 2 (T cell signal) and signal 3 (innate immune receptors such as TLR) are each required [73, 74, 150].

However, the effect of CpG ODN is not clear in T-independent naïve B cell activation. There are studies that suggest that multiple signals (e.g BCR) are required for naïve B cell activation by CpG ODN [54], while others have shown that direct TLR stimulation is sufficient to stimulate the survival and effector functions of naive B cells, notably IgM secretion and cytokine secretion. Recent studies show that purified human naïve B cells express TLR9 and respond significantly (proliferation, IL-6 and IL-10) to CpG ODN [75, 76, 151].

1.5.2 Cytokine secreting B cells

The main known function of B cells is their ability to produce antibodies against specific antigens and their capacity to take up antigen through their BCR and present it via MHC class II, similar to APC. However, B cells also have the ability to secrete various cytokines and modulate the activities of other immune cells, thereby influencing immune responses.

1.5.2.1 Effector B cells

There is substantial evidence that B cells do not act solely through their antibody secretion but also influence the course of immune responses through antibody-independent mechanisms. For example, B cells can be activated by TLR agonist alone and secrete pro-inflammatory or anti-inflammatory cytokines. It has been proposed that B cells can be subdivided on the basis of the cytokines they produce. B cells that have the ability to secrete IL-10 or TGFβ can be classified as regulatory B cells [152, 153] and these are analogous to regulatory T cells [154]. Similar to T cells, B cells can be classified as effector B (Be) cells [155], including Be-1 cells that secrete cytokines such as IFNγ, IL-12 and TNFα and Be-2 cells that secrete cytokines such as IL-2, IL-4, IL-6 and TNFα [153]. Similar to T cells, B cells primed by Th1 cells and antigen produce type I cytokines such as IFNγ and IL-12, whereas B cells primed by Th2 cells and antigen produce IL-1, IL-13 and IL-4. These cytokine-secreting cells have been found in mice infected with specific pathogens. Mice infected with Th1 type pathogens have IFNγ- and IL-12-secreting B cells [156, 157] while mice infected with parasites inducing Th2 immunity have IL-4-secreting B cells [158]. These particular B cells subsets have also been found in human peripheral blood and tonsils [159-161].

1.5.2.2 Regulatory B cells

Immune responses elicited by a pathogen, allergen or foreign antigen need to be controlled to prevent immunopathology. One of the proposed regulatory mechanisms that is currently a subject of intense investigation is a subset of T cells with regulatory functions, namely regulatory T cells (T_{regs}). T_{regs} are functionally distinct T cells and have the phenotype $CD4^+CD25^+Foxp3^+$ cells (natural T_{regs}), or IL-10 producing T regulatory 1(Tr1), or TGFβ1 producing T regulatory 3 (Th3). They have been shown to have suppressive characteristics such that they down-modulate immune responses. Similar to T_{regs}, B cells with regulatory functions (B_{regs}) have been proposed. B_{regs} perform their suppressor role by secreting IL-10 and TGFβ, which suppresses inflammation and they can also interact with other immune cells. The first report about the existence of B_{regs} was by Janeway and colleagues in a murine model of EAE [162]. They found that genetically modified mice that were deficient in B cells developed more severe EAE, and that restoring the B cells in these mice allowed complete recovery from the disease. Since then there have been numerous reports in chronic inflammation models proposing that B_{regs} play a role in dampening immune inflammation and inducing immune tolerance, similar to their T cell counterpart [163]. In models of severe inflammatory bowel disease (IBD), using a B cell-deficient TCRα double KO (am DKO) mice, B_{regs} have been shown to contribute to the suppression of the ulcerative colitis like disease [164]. B_{regs} have also been shown to be involved in diseases such as lupus and experimental rheumatoid arthritis (RA), wherein pathogenic B cells secrete auto-antibodies that play a major role in the disease [162, 165, 166]. All available reports suggest that B_{regs} are induced during chronic inflammation but are not present in normal (healthy) tissues.

CHAPTER 2: RATIONALE, HYPOTHESIS AND OVERALL OBJECTIVE AND AIMS

2.1 Rationale and hypothesis

The IIS has a conserved network of receptors called PRR that have the ability to recognize PAMPs as danger signals. On recognition of their respective ligands, PRR are capable of inducing a variety of immune responses that range from production of cytokines to activation of a variety of cells and resulting in triggering of the AIS. The TLR constitute one family of PRR which provide a sensory mechanism for the detection of infectious threats [167]. Recognition of bacterial DNA or CpG DNA occurs through TLR9 [41]. Upon stimulation with CpG ODN, cells that express TLR9, including pDC and B cells produce Th1-like pro-inflammatory cytokines and upregulate co-stimulatory molecules. Most of these observations were made using cells from blood and spleen.

Unlike blood and the LN, the intestinal tract is constantly exposed to microbes which express a variety of PAMPs, including TLR agonists. We are interested in TLR9 responses in PP, which are the major sites where induction of immune responses occurs in the intestine. In addition to constant exposure to microbial flora, several other factors in the intestinal microenvironment suggest that the response to CpG ODN of immune cells from PP may be different from immune cells in other tissues: (i) unlike peripheral blood and lymph nodes, B cells comprise a large proportion of follicular lymphoid cells (up to 80%) in the PP of sheep [114]; (ii) DC isolated from PP of mice prime Th2 responses while those from spleen prime Th1/Th0 responses [168, 169]; (iii) the intestinal microenvironment may modulate receptor expression in cells, and consequently their capacity to respond to CpG. For example, human MΦ isolated from the lamina propria do not express CD14 and respond poorly to LPS [126], possibly because the intestine is constantly exposed to LPS. Based on these observations, my hypothesis is that:

Innate immune responses to the TLR9 agonist, CpG ODN, in cells from PP are attenuated compared to cells from other tissues such as the blood or lymph nodes. This is due to regulatory mechanisms unique to the intestinal microenvironment, which downregulate TLR responses.

2.2 Overall Objective

Our overall objective was to define the mechanisms which regulate TLR-induced responses in PP. To achieve this objective, we had the following aims.

2.3 Aims

1. Evaluate innate immune responses induced by various CpG ODN classes in immune cells from lymphoid tissues of sheep.

2. Assess responses of PP cells to various TLR agonists alone and in combination.

3. Assess TLR expression in PP.

4. Determine the role of IL-10 and IL-10-secreting PP $CD21^+$ B cells in regulating TLR9-induced responses in PP.

5. Assess the capacity of PP $CD21^+$ B cells to respond to CpG ODN stimulation.

6. Investigate the TLR9 signaling in CD21+ B cells from PBMC and JPP by using the kinome analysis.

CHAPTER 3: INNATE IMMUNE RESPONSES INDUCED BY CLASSES OF CpG ODN IN OVINE LYMPH NODE AND BLOOD MONONUCLEAR CELLS

3.1 Abstract

CpG-ODN signal through Toll-like receptor 9 (TLR9) and trigger a cascade of events that lead to activation of innate and adaptive immune responses. Our current understanding of the immunobiology of host responses to CpG is based largely on studies on peripheral blood mononuclear cells (PBMC) and splenocytes. Little is known regarding CpG-induced responses in other lymphoid tissues. In the present study, we investigated responses induced by CpG in both PBMC and lymph nodes. Cells were isolated from the superficial cervical lymph node (LN) and blood and then stimulated with CpG ODN (either A-, or B- or C-Class ODN). Cytokine production was assayed by ELISA, and lymphocyte proliferation was determined by ^{3}H-thymidine incorporation. NK-like cytotoxicity was analyzed by lysis of ^{51}Cr-labelled target cells. All three classes of CpG induced IFNα and IFNγ in LN cells. In contrast, only A- and C-class ODN induced IFNα and IFNγ in PBMC. Moreover, the IFN levels in LN were 20-40 fold higher than in PBMC. Furthermore, all classes of ODN induced higher IL-12 levels in LN (5-6 fold) than in PBMC. Both B- and C-class ODN induced significant proliferative responses in PBMC and LN, but the A-class ODN did not induce proliferation of PBMC and only induced moderate proliferation of LN cells. A-class ODN induced significant NK-like activity in LN. Thus, all three classes of CpG ODN induced similar responses in LN, and these responses were consistently higher than in PBMC. These observations indicate that CpG ODN-induced responses differ between blood and lymph nodes, and suggest that the functional classification of CpG ODN based on PBMC responses may not be applicable to cells from other immune tissues.

3.2 Introduction

The innate immune system (IIS) has a conserved network of receptors called pathogen recognition receptors (PRR) that have the ability to recognize pathogen associated molecular patterns (PAMPs) as 'danger' signals [3]. On recognition of their respective ligands, PRR are capable of inducing a variety of immune responses. TLR constitute one family of PRR. Currently, thirteen TLR have been identified in mammals and they provide a sensory mechanism for the detection of infectious threats [167, 170]. For example, TLR3, TLR4, TLR7/8 and TLR9 recognize double-stranded RNA, lipopolysaccharides (LPS), single stranded RNA/imidazoquinolines and CpG or bacterial DNA respectively [41, 171-174].

CpG dinucleotides are present at the expected frequency (1/16 base pair) in bacterial DNA. In contrast, the frequency of CpG is suppressed to about one quarter of the predicted value in vertebrate DNA and also the cytosine is often methylated [175]. Based on these differences, it has been proposed that vertebrates have evolved to recognize CpG DNA as a mechanism of detecting the presence of pathogens and stimulating the IIS. Bacterial DNA, but not vertebrate DNA can stimulate mammalian immune cells [54]. Synthetic oligonucleotides containing CpG motifs mimic the immunostimulatory effects of bacterial DNA [54].

Both *in vitro* and *in vivo* studies have demonstrated that CpG ODN are potent activators of the IIS in numerous species including humans, non-human primates, mice, cattle, sheep, pigs, horses, dogs, cats, chickens and fish [53, 56, 176-181]. CpG ODN have been shown to be protective against a variety of pathogens including bacteria, viruses and protozoa in numerous animal models [182-188]. When given with an antigen, CpG has the ability to enhance antigen specific immune responses and this has been shown in humans and numerous animal species [189-194].

Numerous *in vitro* studies have revealed that in mice, a variety of cell types are activated directly or indirectly by CpG ODN. These include B lymphocytes, monocytes, macrophages, dendritic cells, NK cells and even mast cells and, depending on the cell type activated they proliferate, upregulate MHC class I and II, B7-1 and B7-2 co-stimulatory molecules, or express a broad range of cytokines including IL-1, IL-6, IL-

10, IL-12, IFNα, IFNγ and TNFα [24, 195-198]. In humans, plasmacytoid dendritic cells (pDCs) and B cells express TLR-9 and are directly stimulated by CpG ODN to proliferate, produce cytokines and upregulate co-stimulatory molecules [36, 85, 199]. There have been relatively few studies in ruminants, but those that have been reported indicate that CpG ODN induce IFNα and IFNγ secretion in bovine PBMC in vitro, but no NK-like cytotoxicity [200, 201]. It has also been reported that CpG ODN induced IL-6, IL-12 and IFNγ in bovine PBMC whereas B lymphocytes produced only IL-6 [202]. However, CpG ODN stimulation of ovine PBMC induced IFNα and NK-like cytotoxicity but no IFNγ secretion [200, 201], whereas stimulation of ovine lymph node cells induced both IFNα as well as IFNγ [203]. However, in these studies, the capacity of CpG to induce IL-12, a cytokine that plays a key role in the development of Th-1 type immune response, was not evaluated [200, 201].

Three distinct classes of CpG ODN have been characterized: (i) A-class ODN (phosphodiester/phosphorothioate backbone with a poly G tail) trigger maturation of antigen-presenting cells (APC) and directly induce the secretion of high levels of IFNα from pDCs but little or no B cells activation [24, 48, 204], (ii) B-class ODN (multiple CpG motifs on a phosphorothioate backbone) trigger B cells to proliferate and induce little or no IFNα [199, 205, 206], and (iii) C-class ODN (Hexameric CpG motif [5'-TCGTCGTT-3'] linked by a T spacer to GC-rich palindromic sequences) have the functional properties of both A- and B- class ODN [199, 207]. Most of these observations were made using human PBMC or mouse splenocytes. Little is known about the immunostimulatory effects of these ODN in other lymphoid tissues. A recent study indicated that the biodistribution of CpG is more dynamic than previously thought [208]. These investigators showed that following subcutaneous injection of CpG ODN, approximately 10-15% of the ODN were present in lymph node and organs after four hours whereas in serum, the level of the ODN was very low [208]. They also observed a significant increase in ODN concentration between 4 hrs and 24 hrs in the lymph nodes. The accumulation of ODN in lymph nodes suggests that this may be an important tissue in the development of CpG-induced effects *in vivo*. We recently reported that A-class CpG ODN (2216), but not B-class (2007) induced IFNα in PBMC of sheep but both

ODN induced IFNα in lymph node cells (LN) [203]. However, in these previous studies, the newly reported C-class ODN were not evaluated. Sheep provide a convenient model for such studies since an adequate number of cells can be obtained from a number of different immune compartments in the same animal. Furthermore sheep have been used extensively as a model for immunological and physiological studies as reviewed recently [209].

The objectives of this study were to investigate and understand the *in vitro* immunostimulatory effects of the C-class CpG ODN and compare these responses with the other two classes of CpG ODN in PBMC and LN using a variety of immune assays.

3.3 Materials and methods

3.3.1 Oligodeoxynucleotides

Both A-class CpG ODN 2216 and B-class CpG ODN 2007 have previously been shown to be biologically active in sheep in vitro and in vivo [210, 211]. However the immunostimulatory effects of C-class CpG ODN have not yet been evaluated in sheep. Table 3.1 shows the sequences of the ten ODN used in these studies. CpG ODN 2007, 20620 and 2216 were obtained from Merial limited (Lyon, France), while CpG ODN 2006, 2429, 5482 and 8954 were provided by Coley Pharmaceutical group (Wellesley, MA, USA). Control non-CpG ODN 2137, 2243 and 2007GC were purchased from Operon (Alameda, CA, USA).

Table 3.1: A, B and C-class CpG ODN sequences and backbone structure

ODN name	Class	Sequence
2007	B	tcgtcgttgtcgttttgtcgtt
2007GC	B	tgctgcttgtgcttttgtgctt
2006	B	tcgtcgttttgtcgttttgtcgtt
2137	B	tgctgcttttgtgcttttgtgctt
2216	A	ggGGGACGATCGTCgggggg
2243	A	ggGGGAGCATGCTGgggggg
8954	A	ggGGACGACGTCGTGgggggG
2429	C	tcgtcgttttcggcggccgccg
5482	C	tcgtcgtttttcgtgcgttttt
20620	C	tcgtcgttttaCGgcgccgtgccg

Lower case – phosphorothioate backbone
Upper case – phosphodiester backbone

3.3.2 Animals

Suffolk sheep of either sex (2-4 months of age) were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon,

SK, Canada). The animals were housed at the Vaccine and Infectious Disease Organization (VIDO) animal facility and fed ad libitum on a ration of rolled barley and alfalfa hay. All experiments were carried out according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Experimental protocols were approved by the University of Saskatchewan Animal Care Committee. All animals were housed in the same pen throughout each experiment.

3.3.3 Isolation of peripheral blood mononuclear cells (PBMC) and lymph node cells (LN)

Blood was collected from the jugular vein of sheep in EDTA-treated vacutainer tubes (BD Biosciences, Mountain View, CA, USA) and PBMC were isolated using 54% isotonic Percoll^{TM} (Pharmacia Biotech AB, Uppsala, Sweden), as described previously [212]. Cells were counted using a cell counter (Dual Diluter III, Coulter electronics Ltd, Luton, England) and resuspended in AIM V medium (GibcoBRL, Burlington, ON, Canada) containing 2% fetal bovine serum (FBS; GibcoBRL).

Sheep were euthanised and superficial cervical lymph nodes were removed and placed in ice-cold minimum essential medium (MEM, GibcoBRL) containing the antibiotics 100 IU/mL Penicillin, 100 µg/mL Streptomycin sulfate and 0.25 µg/mL Amphotericin B (Sigma-Aldrich, St Louis, Missouri, USA). Cells were isolated from lymph nodes by finely mincing tissue with a scalpel, filtering the cell suspension through a 40-µm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washing cells with PBS (pH 7.2), as described previously [213]. Viable LN numbers were determined by trypan blue dye exclusion and counting in a hemocytometer with the aid of a light microscope. Cells were resuspended in AIM V medium containing 2% FBS.

3.3.4 Tissue culture conditions

Stimulation of PBMC and LN cells was performed in 96-well, round bottom plates (Nunc, Naperville, IL, USA) using AIM V medium supplemented with 2% FBS, 100 IU/mL Penicillin, 100 µg/mL Streptomycin sulfate, 0.25 µg/mL Amphotericin B, 2

mM L-glutamine, 50 μM 2-Mercaptoethanol and 10 μg/mL Polymyxin B Sulfate (Sigma-Aldrich). For measuring cytokines in culture supernatants, PBMC and LN were incubated with either 0.07, 0.22, 0.66, 2.0, 6.0 or 18.0 μg/mL of each of the ten ODN for 48 hrs at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Forty eight hours was previously shown to be the optimal time for detection of cytokine responses in ovine PBMC stimulated with CpG ODN [200]. For each treatment, $5x10^5$ cells were cultured in triplicate wells in 200 μL total volume. Culture supernatants were harvested and stored at -20^oC until assayed for IFNα, IFNγ and IL-12.

3.3.5 Enzyme-linked immunosorbent assay (ELISA) for IFNα, IFNγ and IL-12

The IFNα, IFNγ and IL-12 ELISA assays used during this study were previously shown to detect ovine and bovine IFNα, IFNγ and IL-12 cytokines as described elsewhere [210, 213, 214]. Briefly, polystyrene microtiter plates (Immulon 2; Dynex Technology INC, Chantilly, USA) were coated with capture antibody. For the IFNγ ELISA, mouse anti-bovine IFNγ antibody (clone 2-2-1A) [213] and for IFNα ELISA, two mouse anti-bovine IFNα antibodies (clones IFN-A2 and IFN-A4) were used in coating buffer [215]. For IL-12, mouse anti-recombinant bovine IL-12 antibody (Serotec MCA 1782Z, NC, USA) was diluted to 8 μg/mL in coating buffer. Plates were washed with Tris buffer saline/0.01% Tween 20 (Sigma-Aldrich) (TBST) at all steps. Recombinant bovine IFNγ (bIFN-γ), bovine IFNα (bIFN-α) (Ciba Giegy) and recombinant human IL-12 (rHuIL-12) (Serotec PHP 100) were used as standards. Standards and samples were diluted in TBST/0.5% gelatin (Sigma-Aldrich) (TBST-g) and added to the wells. To detect bound cytokine, rabbit anti-bovine IFNγ antisera or rabbit anti-bovine IFNα anti-sera and mouse anti-bovine IL-12 CC326 biotin (Serotec MCA 2173B; biotinylated at VIDO) were added. Biotinylated goat anti-rabbit IgG (Zymed, South San Fransisco, CA, USA) and strepavidin-alkaline phosphatase (GibcoBRL) were used for detection. The assay was developed by using p-nitrophenyl phosphate (Sigma-Aldrich) substrate in 1 % diethanolamine (Sigma-Aldrich) and 0.5 mg/mL magnesium chloride. The reaction was stopped by adding EDTA to each well.

Optical density of the reaction product was measured at 405 nm using a 490 nm reference on a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Sample concentrations were calculated using Microplate Manager 5.0.1 version software (Bio-Rad).

3.3.6 Cytotoxicity assay

A NK-sensitive target cell line (K562 cells), which was previously shown to be sensitive to cytotoxicity by ovine PBMC were prepared as described by Mena *et al* [200]. Briefly, NK-sensitive target cells were labeled, washed and resuspended in culture medium. Cells (1×10^4) were added to each well in a round bottom 96-well plate. All assays were performed in triplicate and six wells per plate were used to determine the mean spontaneous release (release of ^{51}Cr from K562 cells in media alone) and total release (lysis of K562 cells with 5% Triton X-100). One million effector cells (PBMC) were added per well for a 100:1 effector to target ratio. LN or PBMC and K562 cells were co-cultured for 24 hrs at 37°C, either in medium alone, stimulated with 15 ng/mL of recombinant bovine IL-2 (bIL-2, Ciba Giegy, Basel, Switzerland), or incubated with 2 μg/mL of either of the three classes of ODN in a final volume of 200 μL. Supernatants were harvested using a supernatant collection system (Skatron, Virginia, USA) and counts per minute (c.p.m.) were determined using a gamma counter (Model 5500, Beckman Instruments). Spontaneous release was always less than 25% of total release. The percentage cytotoxicity was calculated using the formula {(experimental c.p.m.) – (spontaneous c.p.m.) / (total c.p.m.) – (spontaneous c.p.m.)} x 100.

3.3.7 Lymphocyte Proliferative responses (LPR)

PBMC and LN resuspended in culture medium for LPR assays were pulsed with 0.4 μCi [^3H] thymidine (Amersham Pharmacia, Piscataway, NJ) during the final 6 hrs of a 72-hrs incubation as previously described [200]. Cells were harvested using standard

liquid scintillation protocols and uptake of ^{3}H-thymidine was assessed in a beta counter (Topcount, Packard Instrument Company, Meriden, CT). The LPR were calculated as the mean counts per minute (c.p.m) of triplicate cultures and expressed as a stimulation index (c.p.m in the presence of stimulus/c.p.m in the absence of stimulus).

3.3.8 Statistical analysis

Data were analyzed using the statistical software program SPSS 12 (SPSS Inc., Chigaco, IL, USA). Data that were not normally distributed were transformed by ranking. The differences among individual groups were examined by performing one-way analysis of variance (ANOVA). If the results of ANOVA were significant ($p< 0.05$), the means of the transformed data were compared using Tukey's test. The residuals of each ANOVA were examined to determine that the assumptions of the analysis had not been violated. Differences were considered significant when $p<0.05$.

3.4 Results

3.4.1 Effect of CpG ODN class and concentration in PBMC and LN

3.4.1.1 IFNα induction in LN and PBMC

Both A-class (2216, 8954) and C-class (2429) CpG ODN induced IFN*α* secretion in PBMC (Fig. 3.1 A) and in LN (Fig. 3.1 B). B-class ODN (2006, 2007) also induced secretion of IFN*α* at 0.66, 2.0, and 6.0 μg/mL of ODN but only in LN and not in PBMC (Fig. 3.1 A-B). IFN*α* levels were 30-40 fold higher in LN than in PBMC (Fig. 3.1 A-B). Not only was the amount of IFN*α* higher in CpG-stimulated LN but also more ODN sequences induced the LN to produce IFN*α* (Fig. 3.1 A-B). There was an effect of CpG concentration on the IFNα response (Fig. 3.1 A). At low concentration (0.07 – 0.22 μg/mL), CpG ODN induced small amounts of IFN*α*. Production of IFN*α* continued to increase as the concentration of ODN increased (0.66-2.0 μg/mL), and reached a plateau (2.0 -18.0 μg/mL). Thus the optimal dose appears to be 2.0 μg/mL. Control GpC ODN did not induce IFNα secretion in PBMC or LN, indicating that IFNα secretion by either PBMC or LN was 'CpG- specific'.

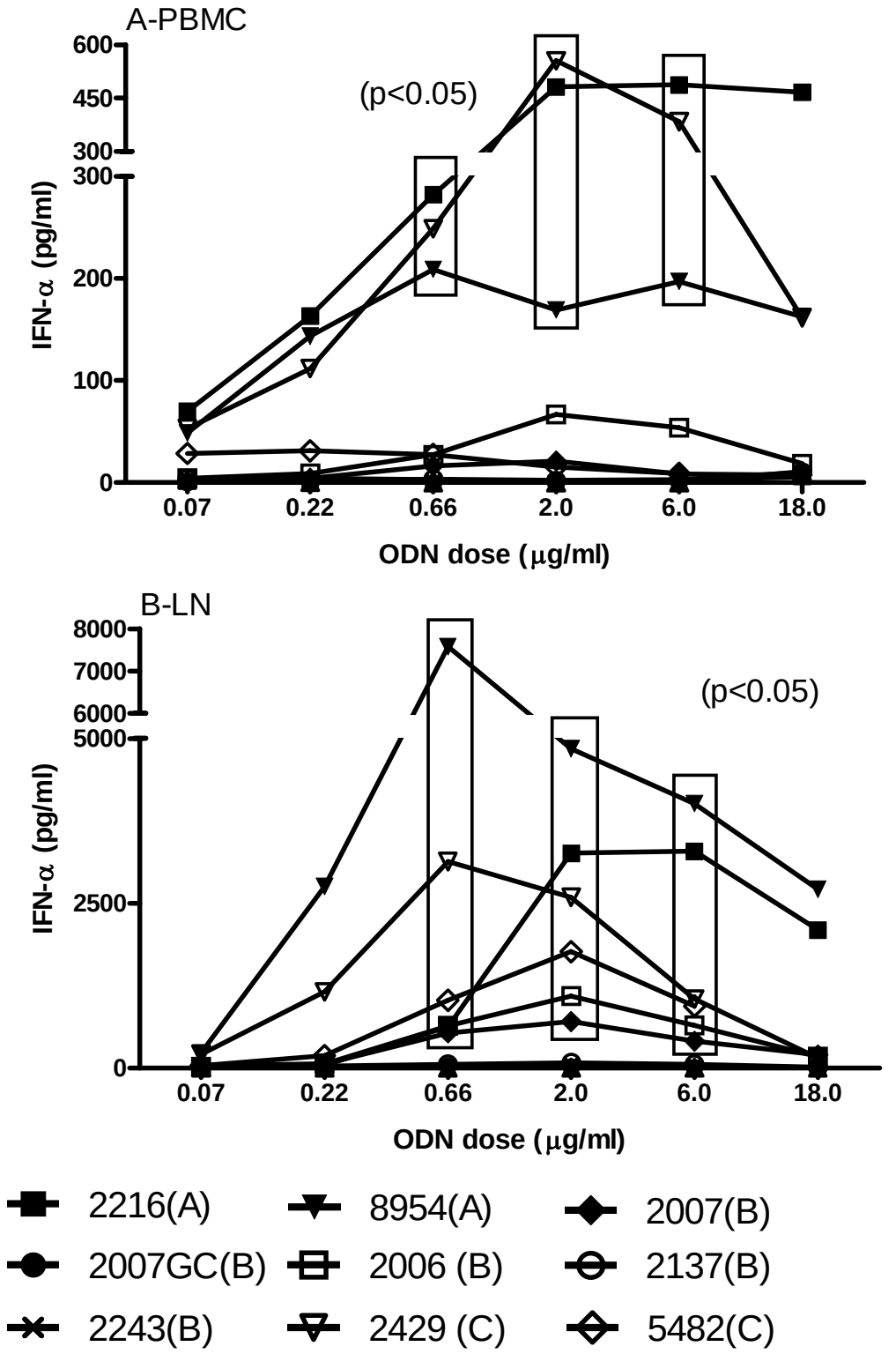

Fig. 3.1 A-B: IFNα secretion by ovine PBMC and LN following stimulation with varying concentration of CpG ODN. Data represent the mean of 8 animals (n=8) for each concentration of CpG ODN. Boxed ODN responses are similar but significantly different ($p<0.05$) from ODN responses outside the box. The letter in parenthesis in figure legend indicates the class of ODN.

3.4.1.2 IFNγ induction in LN and PBMC

Production of IFNγ in response to CpG ODN stimulation of PBMC and LN followed a similar pattern to that observed with IFNα. A-class (2216, 8954) and C-class CpG ODN (2429, 5482) stimulation resulted in significant production of IFNγ in both PBMC and LN. However, the level of IFNγ was approximately 20-30 fold higher in LN. Also, it was observed that B-class ODN 2006 and 2007 induced IFNγ in the LN (Fig. 3.2 B) but not in the PBMC (Fig. 3.2 A). However, significant induction of IFNγ by B-class ODN required at least 2.0 μg/mL concentration of CpG ODN (Fig. 3.2 B). The responses were dependent on the concentration of CpG ODN (Fig. 3.2 A-B). At low concentrations of 0.07 to 0.22 μg/mL, CpG ODN induced low amounts of IFNγ but as the concentration increased from 0.66 to 2.0 μg/mL, responses continued to increase and then reached a plateau at 6.0 to 18.0 μg/mL (Fig. 3.2 A-B). Just as for IFNα, the IFNγ response was CpG specific as the non-CpG ODN controls did not stimulate any significant production of this cytokine (Fig. 3.2 A-B).

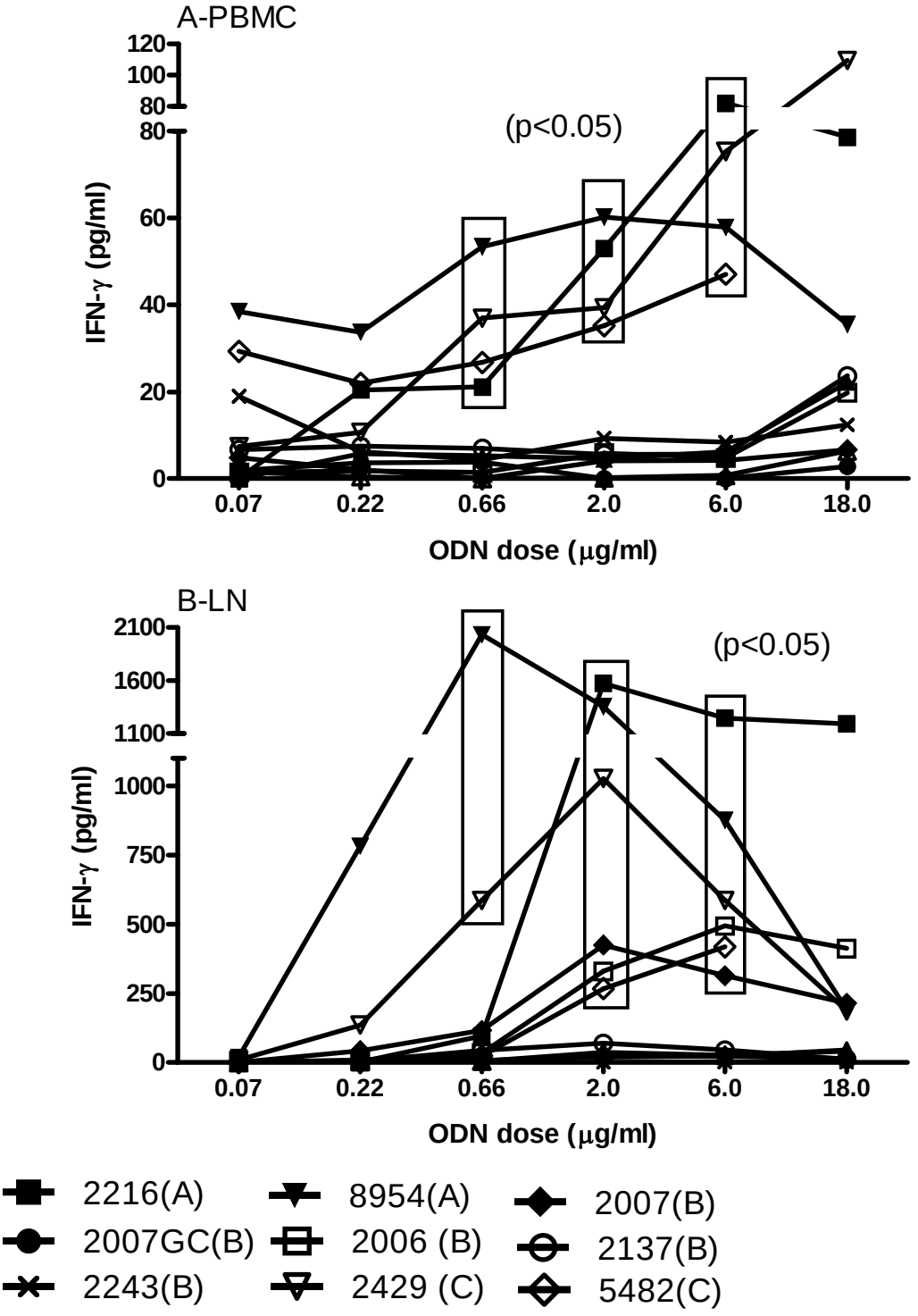

Fig. 3.2 A-B: IFNγ secretion by ovine PBMC and LN following stimulation with varying concentration of CpG ODN. Data represent the mean of 8 animals (n=8) for each concentration of CpG ODN. Boxed ODN responses are similar but significantly different ($p<0.05$) from ODN responses outside the box. The letter in parenthesis in figure legend indicates the class of ODN.

3.4.1.3 Proliferative responses in LN and PBMC

In LN and PBMC, B-class (2007, 2006) and C-class (2429, 5482) ODN induced significant proliferation compared to the control GpC ODN (2007 GC, 2137, 2243) (Fig. 3.3 A-B). In contrast, A-class ODN (8954, 2216) induced significant proliferation in LN at concentration of 2.0 and 6.0 μg/mL of ODN (Fig. 3.3 B) but did not induce strong proliferation in PBMC (Fig. 3.3 A). The proliferation of PBMC and LN stimulated with CpG increased as the concentration of CpG ODN increased (Fig. 3.3 A-B). The highest proliferative responses were seen with ODN in the range of 2.0 to 6.0 μg/mL as was observed for IFNα and IFNγ responses. Also proliferative responses were CpG specific as the control GpC (non-CpG) ODN did not stimulate any proliferative effects in both PBMC and LN (Fig. 3.3 A-B).

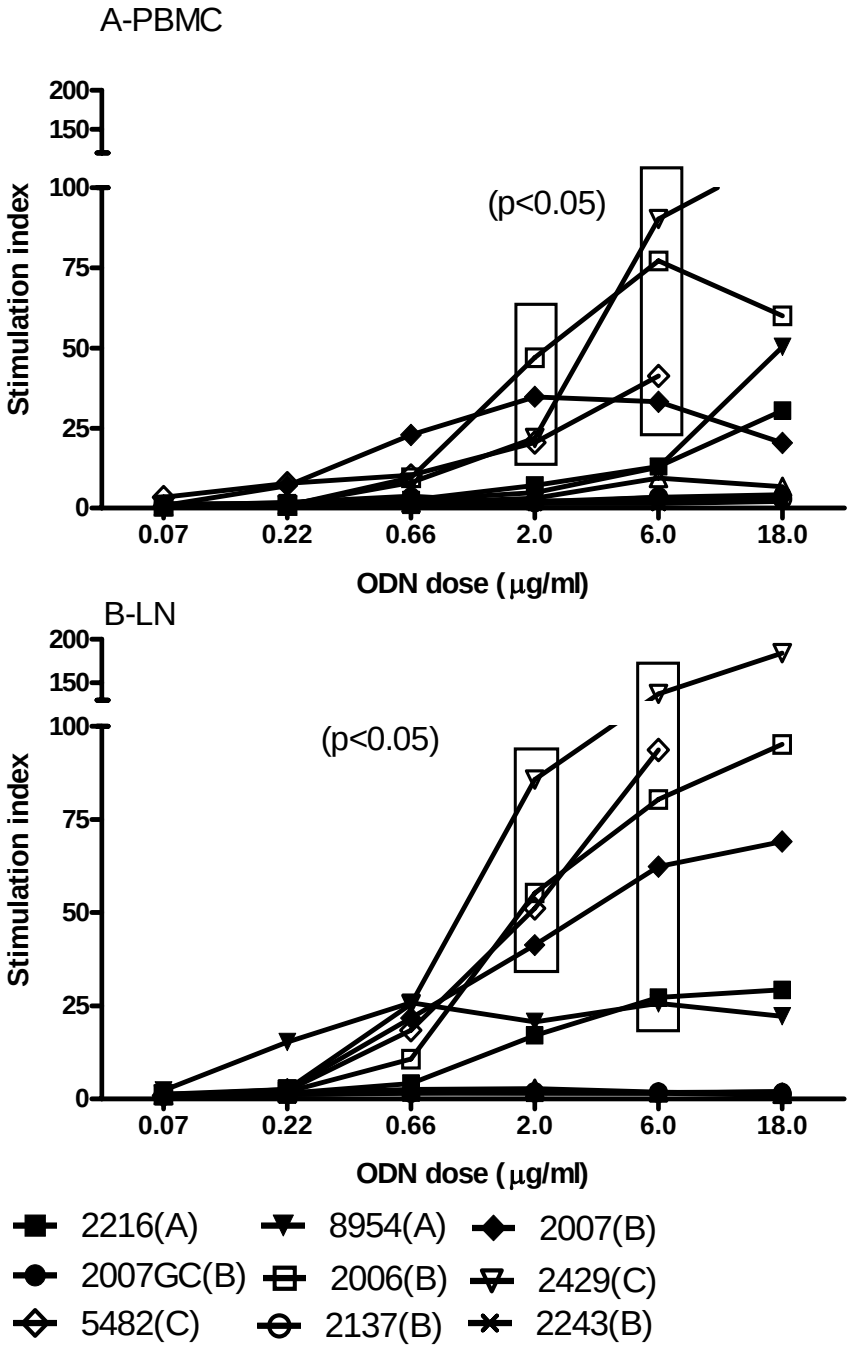

Fig. 3.3 A-B: Proliferative responses by ovine PBMC and LN following stimulation with varying concentration of CpG ODN. Data represent the mean of 8 animals (n=8) for each concentration of CpG ODN. Boxed ODN responses are similar but significantly different ($p<0.05$) from ODN responses outside the box. The letter in parenthesis in figure legend indicates the class of ODN.

3.4.2 IL-12 induction in LN and PBMC

Since the optimal proliferative responses occurred at about 2 μg/mL of CpG ODN, the ability of eight ODN to induce IL-12 in both LN and PBMC was assessed at this concentration. All three classes of CpG ODN (A-class: 8954; B-class: 2006, 2007; C-class: 2429, 20620) induced significant IL-12 secretion in the LN (Fig. 3.4 B). In contrast only B-class (2006) and C-class ODN (2429, 20620) stimulated significant IL-12 secretion in PBMC (Fig. 3.4 A). Induction of IL-12 was 5-6 fold higher in LN than in PBMC (Fig. 3.4 A-B). Again, this response was CpG specific as the GpC controls did not induce any IL-12 in LN and PBMC (Fig. 3.4 A-B). In LN, there was no significant distinction between the stimulatory effects of the three classes of CpG ODN in terms of IL-12 induction whereas in PBMC, only B- and C-class induced significant IL-12.

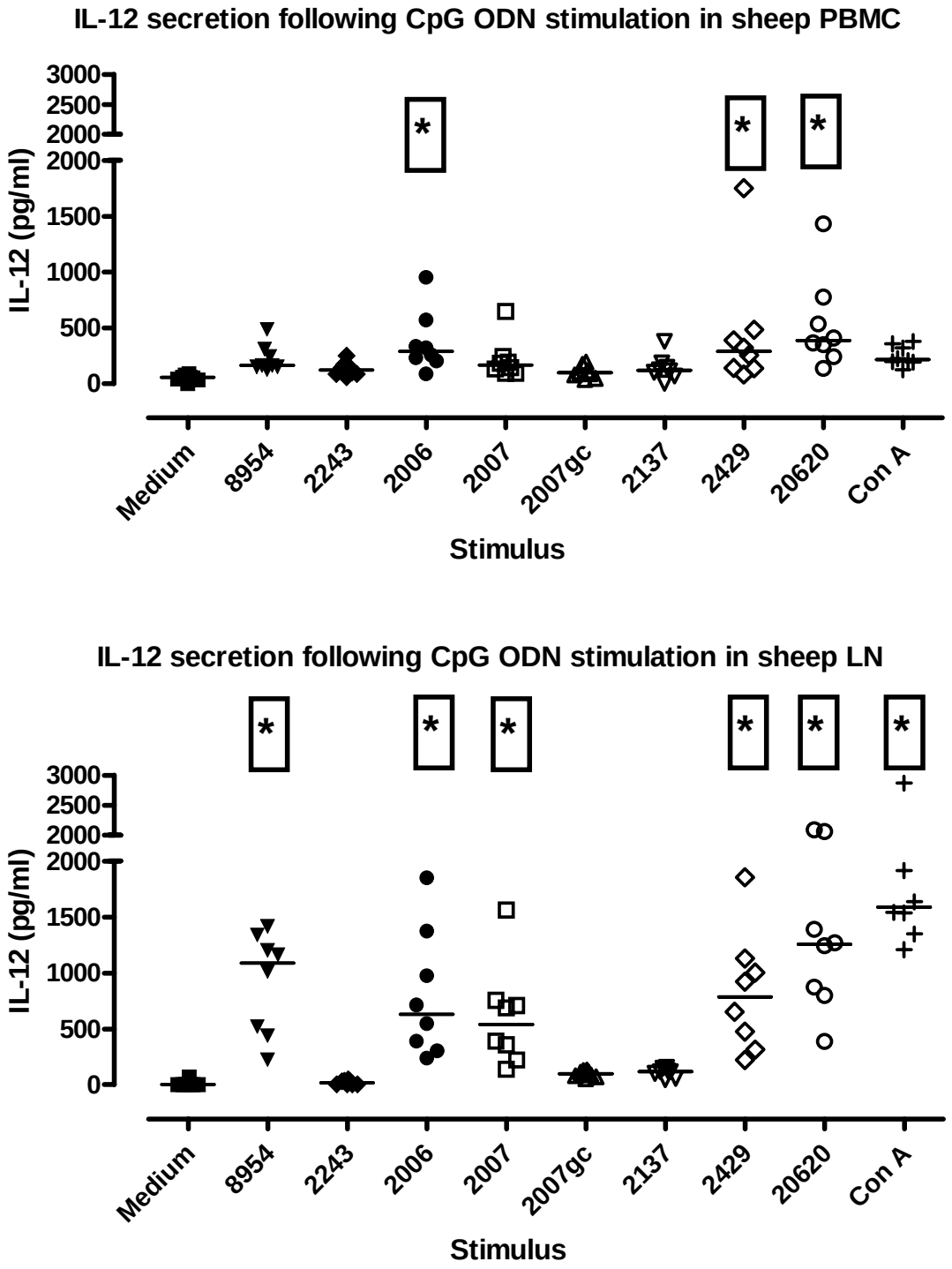

Fig. 3.4 A-B: IL-12 responses in ovine PBMC and LN following stimulation with either 2 μg/mL CpG ODN, 5 μg/mL concavalin A (ConA) or medium alone. Data for individual animals are presented with median value indicated by horizontal bar for each treatment group (n=8). Significant IL-12 secretion relative to GpC control is indicated by a boxed * ($P<0.05$).

3.4.3 NK-like cytotoxicity in LN and PBMC

It is believed that an important characteristic of CpG ODN, especially of the A-class, is the activation of NK cells [48]. Previously, it was shown that A-class and B-class CpG ODN have the ability to stimulate cytolytic activity in ovine PBMC [200] but NK-like cytotoxicity has not been assayed in LN. In the present study, the ability of CpG ODN to stimulate cytolytic activity in ovine PBMC and LN was assessed. It was observed that A-class ODN (8954) induced significant NK-like cytotoxicity in LN (Fig. 3.5 B). Although B (2006, 2007) and C-class (2429, 20620) ODN induced NK-like cytotoxicity in LN, this was not statistically different from the GpC control (Fig. 3.5 B). None of the three classes of ODN [A (8954), B (2006) or C-class (2429, 20620)] stimulated significant cytolytic activity in PBMC when compared to the GpC control (Fig. 3.5 A).

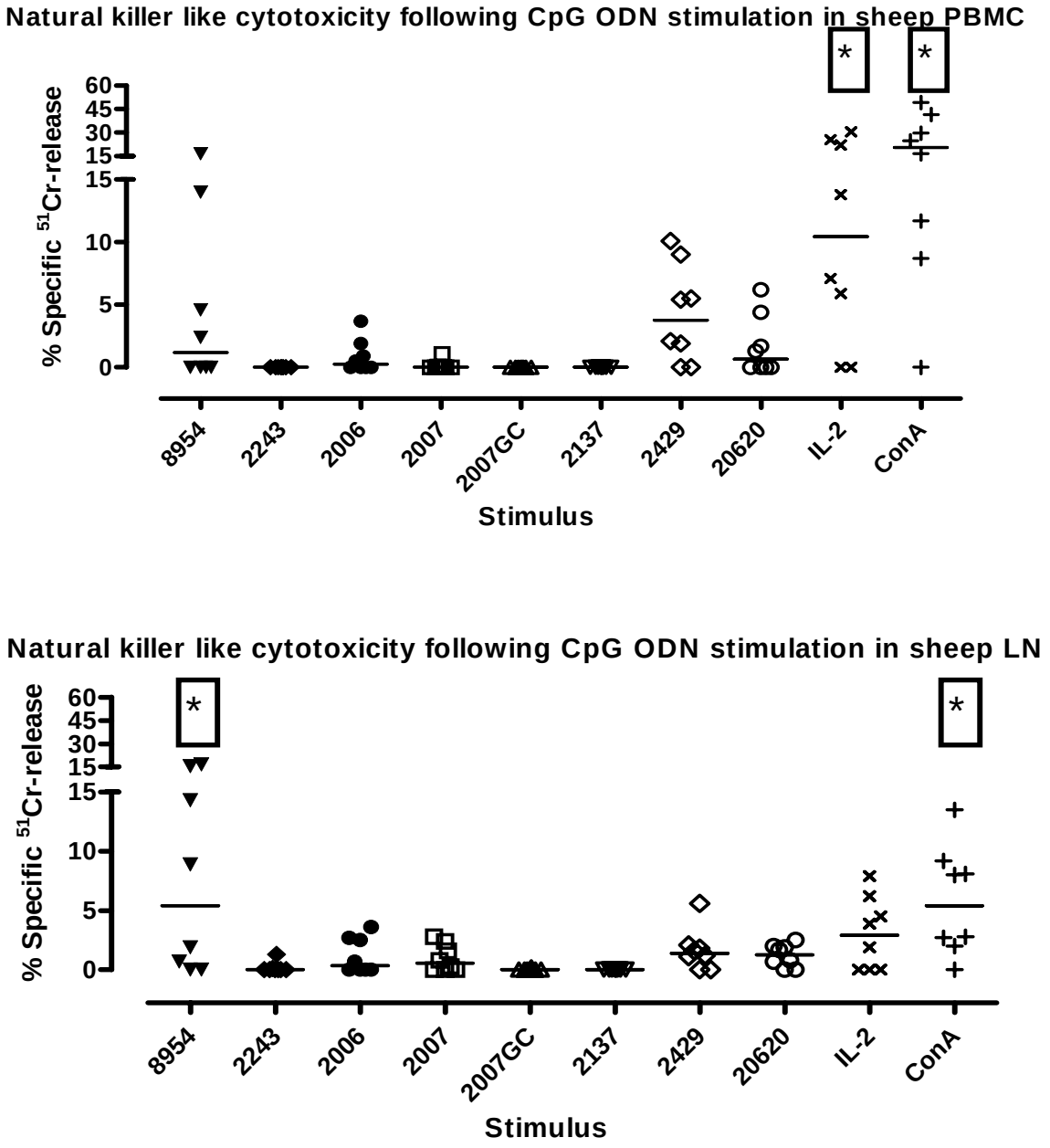

Fig. 3.5 A-B: NK-like cytotoxicity responses in ovine PBMC and LN following stimulation with either 2 µg/mL CpG ODN, 15ng/mL IL-2, concavalin A (ConA) or medium alone. Data for individual animals are presented with median value indicated by horizontal bar for each treatment group (n=8). The effector (PBMC, LN) to target (K562 cells) ratio was 100:1. Significant increases in specific ^{51}Cr-release relative to GpC controls are indicated by boxed * ($P<0.05$).

3.5 Discussion

The present investigation revealed for the first time that C-class CpG ODN are highly stimulatory in ovine PBMC and LN. Furthermore, we showed that while there were differences in ODN class responses in PBMC, all three classes of CpG ODN induced similarly high responses in LN. C-class ODN have the immunostimulatory effects of both A- and B-class ODN in ovine cells. These observations are consistent with studies in humans, mice and Rhesus monkeys where C-class ODN have been shown to have the combined stimulatory effects of A- and B-class ODN [199, 207, 216, 217]. While we observed combined immunostimulatory effects of A- and B-class CpG ODN for the C-class ODN, similar to those reported by others [199, 207, 216, 217], our investigations revealed some new findings. First, CpG-induced responses (IFNα, IFNγ, IL-12 and proliferation) were much higher in LN than in PBMC. Second, while A- and C-class ODN induced significant IFNα and IFNγ in both PBMC and LN, B-Class ODN induced IFNα and IFNγ responses only in LN, but not in PBMC.

The immune stimulatory activities of A- and B-class CpG ODN classes have been described in the sheep PBMC [200]. B-class ODN are potent at stimulating proliferative responses but do not induce significant IFNα in PBMC. In contrast, A-class CpG ODN induce high levels of IFNα and stimulate NK-like cytotoxic activity [200]. In human PBMC and mouse splenocytes, a clear distinction has been demonstrated between A-, B- and C-class in terms of cytokine production (*e.g.* A-class induce mainly IFNα production) and proliferative responses [24, 199, 207].

In LN, B-class ODN induced significant IFNα production and the levels were similar to those seen following stimulation with A- or C-class ODN. This was surprising since most studies have shown that B-class CpG ODN induce little or no IFNα production in PBMC. Thus, our data suggest that B-class ODN are equally efficient in inducing IFNα production in LN. Moreover, B-class ODN induced significant IFNγ similar to that of A and C-class ODN in the LN. These observations are consistent with the results of Nichani and colleagues [203] who observed increased levels of the IFN-induced enzyme 2'5'-A synthetase following subcutaneous injection of B-class CpG ODN 2007. In terms of proliferative effects, both LN and PBMC

showed strong proliferation in response to B- and C-class CpG ODN. However, A-class CpG ODN induced lower but significant proliferation at higher concentrations in LN but not in PBMC.

The biodistribution and metabolism of immunostimulatory ODN 2006 in mice and rats was recently reported [208]. It was observed that upon injection of ODN, the maximal concentration of the CpG ODN in draining lymph node, kidney and liver were around 10-15% of that of the injection site, and that only very low, transient levels of CpG ODN were found in plasma [208]. The accumulation of CpG ODN in draining LN [208] and our observations that B-class ODN are highly stimulatory to LN may explain why B-class ODN have potent immunostimulatory effects *in vivo* [203, 218, 219]. Overall, the data presented here indicate that there are substantial differences in CpG-induced responses between LN and PBMC. The differences for the responses to CpG in the two tissues are not known at the present time, but may be partly due to differences in cell populations that respond to CpG ODN. These differences may involve variations in frequency, and/or activation state of the responder cells. Little is known about CpG ODN-induced responses in cells from other immune compartments like spleen, lung, gut-associated lymphoid tissues (GALT) and other mucosal-associated lymphoid tissues (MALT). Differences in responses between PBMC and LN suggest that cells from these other tissues might show different responses to various classes of CpG ODN. Such studies will be important in targeting CpG ODN to appropriate immune compartments in order to maximize the benefits of CpG treatment.

IL-12 is a pro-inflammatory cytokine that is thought to link innate and adaptive immunity by favoring the differentiation of T helper 1 (Th1) cells and production of IFNγ [220]. In mice, CpG ODN directly activate monocytes, macrophages and dendritic cell which respond by secreting high levels of Th1 promoting cytokines such as IL-12 [221]. In this study, significant IL-12 was induced in ovine PBMC by B- and C-class ODN while in LN all three classes of CpG ODN induced this cytokine. However, the difference between the levels of IL-12 produced by LN in response to different CpG classes was not as dramatic as that produced by PBMC.

Given the importance of NK activation in innate immunity, the ability of all three classes of ODN to induce NK-like cytotoxic activity was assessed. Only A-class

ODN (8954) induced significant CpG specific non-MHC restricted NK-like cytotoxicity. The NK-like cytotoxicity responses were highly variable from animal to animal and likely contributed to the lack of statistical significance. Nonetheless our results are consistent with those reported previously by Mena *et al* [200] for A- and B-class.

The concentration of CpG ODN used can also have a significant impact on the responses observed. For example, highly stimulatory ODN will have activity at lower doses while ODN with low activity will require higher doses. Furthermore very high doses of ODN may result in poor responses. For these reasons, it was important to determine the optimal concentration of each ODN in PBMC and LN. Our observations indicated that the CpG concentrations that induced optimal responses were between 0.66 to 2.0 μg/mL for IFNα, IFNγ and proliferative responses *in vitro*.

Although the new C-class CpG ODN induced innate immunostimulatory responses comparable to A- and B-class CpG ODN, the C-class ODN may offer advantages over A- and B-class ODN. First, C-class ODN have broad immunostimulatory properties, as indicated by their capacity to stimulate potent interferon and B cells responses in PBMC and LN. Second, C-class CpG ODN have phosphorothioate backbone similar to B-class CpG ODN and therefore would not be rapidly degraded *in vivo*. Thus, this new class of CpG ODN has the potential to be used for stimulation of innate and adaptive immunity to protect against infections.

3.6 Conclusion

The immune responses induced by CpG can vary remarkably both quantitatively and qualitatively between cells from different immune tissues. Data from one tissue cannot be extrapolated to other tissues. Therefore, one must determine the effects of specific ODN in tissues of interest.

CHAPTER 4: SHEEP PEYER'S PATCHES RESPOND POORLY TO TLR AGONISTS AND TLR7/8 AGONISTS DOWNREGULATE CpG-INDUCED RESPONSES IN BLOOD MONONUCLEAR CELLS.

4.1 Abstract

Peyer's patches (PP) are secondary lymphoid tissues and are the primary sites for immune induction in the intestine. In our previous study, we demonstrated that there were marked differences between CpG (TLR9 agonist)-induced responses in LN and blood. We wondered whether TLR agonists would stimulate PP cells in a similar manner. We found that cells from both ileal PP (IPP) and jejunal PP (JPP) responded poorly to all the TLR agonists poly(I:C) (agonist for TLR3), LPS (TLR4), single stranded RNA (ssRNA) (TLR7/8) and CpG ODN (TLR9). All the responses tested (IFNα, IFNγ, lymphocyte proliferation, and IgM secretion) were significantly lower in PP compared to LN. We also observed that the levels of mRNA expression for TLR7/8/9 in PP were similar to the levels in LN and PBMC. It has been proposed that simultaneous stimulation with multiple TLR agonists may result in complementary or inhibitory responses. We therefore tested whether simultaneous stimulation with multiple TLR agonists would trigger significant responses in PP and other tissues. We observed no significant increase in TLR-induced responses in PP cells and PBMC upon stimulation with combinations of TLR agonists. However, we observed significant inhibitory responses upon co-administration of TLR7/8 and TLR9 agonists in LN and PBMC. Therefore, we further investigated the effect of TLR7/8 agonists on the TLR9 agonist-induced responses in ovine PBMC and purified blood B cells by simultaneous activation with CpG ODN 2429 and the TLR7/8 agonists; ssRNA oligoribonucleotides (ORN) or imiquimod. Compared to responses induced by CpG alone, simultaneous activation with TLR9 and TLR7/8 agonists resulted in significantly reduced IFNα production, cell proliferation and IgM responses in PBMC. Importantly, the TLR7/8 agonists reduced the CpG-induced proliferative responses in purified blood B cells. Pre-incubation of cells with CpG for 2 or 4 hrs before addition of imiquimod (TLR7/8 agonist) still resulted in reduced CpG-induced proliferation, indicating that the down-

regulatory mechanisms are not associated with competition for cellular uptake or receptors for imiquimod and CpG ODN. Imiquimod reduced the CpG-induced proliferation in a dose-dependent manner but this was not associated with an increase in caspase enzymes or IL-10, implying that the unresponsiveness was not due to activation-induced cell death or suppression by IL-10. Ovine B cells constitutively expressed TLR7, TLR8 and TLR9 mRNA suggesting a possible role of TLR cross-talk in the down-regulatory mechanisms. We conclude that while simultaneous activation with TLR7/8/9 agonists may influence responses in immune cells from blood and lymph nodes, these combinations of TLR agonists had no effect on responses in PP cells.

4.2 Introduction

The innate immune system uses a set of germline-encoded pattern recognition receptors (PRR) to detect pathogen associated molecular patterns (PAMPS) present in microbial pathogens [18]. PRR are strategically located in various cells and have been grouped into different families that include Toll-like receptors (TLRs), Caspase recruitment domain (CARD) helicases, retinoic acid induced gene (RIG)-like receptors (RLR), nucleotide oligomerization domains (NODs), C-type lectins, complement receptors and others [222]. TLRs, the main PRR family, are trans-membrane signaling molecules currently comprised of at least 13 members in mammals named TLR1-TLR13 [18, 167, 222, 223].

TLR3 recognizes viral double stranded dsRNA and poly(I:C) whereas TLR4 detects lipopolysaccharide (LPS). Endosomal TLR7 and TLR8 recognize viral ssRNA and the small antiviral compounds, imidazoquinolines [224, 225], while TLR9 is the receptor for CpG-rich viral and bacterial DNA [226]. Agonists for TLR7/8 and TLR9 are potent activators of innate and adaptive immunity, and have attracted a great deal of attention due to their potential as vaccine adjuvants and immunotherapeutic agents. Activation of TLR7/8 predominantly induces Th1 cytokine and chemokines including IFN-α, IP-10, IL-12, IL-6 and TNF-α [224, 227-230], increased expression of co-stimulatory molecules and upregulation of the early activation marker CD69 [224, 231-233]. Similarly, activation of TLR9 induces predominantly Th1 cytokines, chemokines and increased expression of costimulatory molecules, but also activates B cells to proliferate and secrete IgM and to produce cytokines such as IL-6 and IL-12, [234].

PP have been shown to be the primary sites where immune responses are initiated in the intestine. However, PP are constantly exposed to PAMPs (TLR agonists) by the presence of commensals in the lumen. We reasoned that their response may be quite different from blood and LN. In sheep and many other species including humans, there are two distinct PP in the small intestine namely the jejunum PP (JPP) and ileal PP (IPP), each with their own characteristic development, structure and function [235]. JPP) but IPP are the primary sites where active immunity is induced in the intestine

[110, 236, 237]. Unlike peripheral LN, the major proportion of lymphoid cells in the PP comprise of B cells (up to 80% in JPP and > 90% in IPP) [100, 114] whereas the B cells frequency in PBMC or LN is around 30-40% [238]. In our initial studies, we found that PP cells respond poorly to stimulation with single TLR agonists.

However, experience with TLR9 agonist, CpG ODN suggests that individual TLR agonists in ruminants are not as potent immune activator as suggested in rodent models [239]. Given that viruses and bacteria express several TLR agonists and most likely pathogens stimulate multiple TLRs simultaneously, the consequences of activating multiple TLRs has generated a lot of interest. Evidence is accumulating to support the notion that activation of multiple TLRs can result in complementary, synergistic or antagonistic effects. In this regard, simultaneous activation of dendritic cells (DC) with TLR9 and TLR4 agonists, CpG ODN and LPS resulted in additive effects on IL-12 production [240]. Similarly, costimulation of TLR3 and TLR8, or TLR4 and TLR8 resulted in a synergistic IL-12 response, but costimulation of TLR3 and TLR4 had no effect on the IL-12 response [94]. In contrast, simultaneous activation with agonists for TLR7/8 and TLR9 resulted in inhibition of IFNα responses [241, 242].

An understanding of the cellular events triggered by combinations of TLRs will be valuable in the rational design of more successful TLR-based immunotherapies and vaccination strategies. TLR agonists can be grouped into two main categories based on their dependence or independence on the adaptor molecule MyD88. It has been proposed that co-stimulation with agonists sharing common pathway (MyD88 dependent (D) or independent (I) agonists) do not induce synergistic, but rather may lead to inhibitory responses. In contrast, co-stimulation with a D agonist and I agonist lead to synergistic responses [95].

It is thought that most of the beneficial effects of TLR7/8 and TLR9 activation are mediated primarily through IFN-α produced by DC [43, 243]. Consequently, studies exploring costimulation of these TLRs have focused primarily on IFNα responses in DC, and relatively less is known about the effects of costimulation of other cells. B cells are one of the major cell populations which can be significantly influenced through TLR activation. Many B cells express TLR7/8/9 and, activation of TLR9 signal in B cells can synergize with the antigen-specific BCR signal resulting in enhanced antibody

production [43]. More recently, it was suggested that in addition to $CD4^+$ T-cell help, generation of T-dependent antigen-specific antibody responses requires activation of TLRs in B cells [148]. Thus, TLR activation can have dramatic effects on the behavior of B cells, but not much is known about multiple TLR stimulation in these cells. One recent report suggested that simultaneous activation with TLR7 and TLR9 agonists had no effect on human B-cell responses [242].

It has been suggested that the use of combinations of TLR agonists may be necessary to induce strong immune responses in large animal [239]. However, the consequences of multiple TLR stimulation have not been explored in ruminants. Given that TLR responses can differ dramatically from one species to another, we decided to investigate effects of co-stimulation of TLR3, TLR4, TLR7/8 and TLR9 in sheep.

The objectives of the present studies were (i) to determine whether TLR agonists and their combinations can induce innate immune responses in ovine immune cells (PP, LN and PBMC), and (ii) to assess innate immune responses in ovine blood mononuclear and B cells following co-stimulation with TLR7/8 and TLR9 agonists.

We report that co-stimulation of ovine blood mononuclear and B cells with TLR7/8 and TLR9 agonists results in significant reduction in ensuing innate immune responses.

4.3 Materials and methods

4.3.1 TLR agonists and Animals

The TLR7/8 agonists used included synthetic ssRNA oligonucleotide (ORN) R-1075 [224] obtained from Coley Pharmaceutical Group (Ottawa, ON, Canada) and a synthetic imidazoquinoline compound imiquimod purchased from Invivogen (San Diego, CA, USA). CpG oligodeoxynucleotide (ODN) C-class ODN 2429; sequence; tcgtcgttttcggcggccgccg [244] or B-class CpG ODN 2007; sequence tcgtcgttgtcgttttgtcgtt were obtained from Merial Limited (Lyon, France). Suffolk sheep of either sex (2 to 4 months of age) were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). The animals were housed at the Vaccine and Infectious Disease Organization (VIDO) animal facility and fed ad libitum on a ration of rolled barley and alfalfa hay. All experiments were carried out according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Experimental protocols were approved by the University of Saskatchewan Animal Care Committee. All animals were housed in the same pen throughout each experiment.

4.3.2 Isolation of PBMC, JPP, IPP and LN cells

Blood was collected from the jugular vein of sheep in ethylene di-amine tetra-acetic acid (EDTA)-treated vacutainer tubes (BD Biosciences, Mountain View, CA, USA) and PBMC were isolated using 54% isotonic Percoll^{TM} (Pharmacia Biotech AB, Uppsala, Sweden), as described previously [201, 212]. Cells were counted using a cell counter (Dual Diluter III, Coulter electronics Ltd, Luton, England) and resuspended in AIM-V medium (GibcoBRL, Burlington, ON, Canada) containing 2% FBS (GibcoBRL).

Sheep were euthanised and superficial cervical and mesenteric lymph nodes were removed and placed in ice-cold minimum essential medium (MEM, GibcoBRL) containing the antibiotics 100 IU/mL Penicillin, 100 µg/mL Streptomycin sulfate and

0.25 μg/mL Amphotericin B (Sigma-Aldrich, St Louis, Missouri, USA). Cells were isolated from lymph nodes by finely mincing tissue with a scalpel, filtering the cell suspension through a 40-μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washing cells with phosphate buffered saline calcium and magnesium free (PBSA) (pH 7.2), as described previously [213]. JPP and IPP tissues were also removed and placed in MEM (GibcoBRL) containing antibiotics (100 IU/mL Penicillin, 100 μg/mL Streptomycin sulfate, 0.25 μg/mL Amphotericin B) (Sigma-Aldrich). The JPP and IPP cells were also isolated from the same sheep as described previously [110, 245]. The number of viable cells in all tissues was determined by trypan blue dye exclusion and counting with a hemocytometer under a light microscope. Cells were resuspended in AIM V medium containing 2% FBS.

4.3.3 Magnetic activated cell sorting (MACS)

The $CD21^+$ B cell fraction of PBMC was isolated as previously described [246] with minor modifications. Briefly PBMC were incubated with mouse anti-bovine CD21 antibody (IgG1, AbD Serotec, UK) for 15 min at 4 °C. The cells were then washed twice with magnetic activated cell sorting (MACS) buffer (PBSA, 0.5M EDTA and 10% BSA) by spinning for 8 min at 440 ×g and incubated with goat anti-mouse IgG1 phycoerythrin (PE) conjugate (Southern Biotech, AL, USA) for 15 min at 4 °C. The cells were then labeled with anti-PE magnetic beads for 15 min at 4°C and eluted through the LC MACS column (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions. The $CD21^+$ fraction was flushed out, washed in PBS and re-suspended in AIM V media containing 2% FBS. The purity of the CD21+ cells were above 93%.

4.3.4 Tissue culture conditions and stimulation with TLR agonists

Cells were re-suspended in AIM V medium supplemented with 2% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 2mM

L-glutamine, 50 μM 2-mercaptoethanol and 10 μg/mL polymyxin B sulfate (Sigma-Aldrich) and added to 96-well, round bottom plates (Nunc, Naperville, IL, USA). For each treatment, $5x10^5$ cells were cultured in triplicate wells in 200 μL total volume. Cells were incubated at 37°C in a 5% CO_2 atmosphere and 95% humidity. Optimal cell stimulation concentrations for TLR3, TLR4, TLR7/8 or TLR9 agonists were established previously [200, 247]. Cells were stimulated with TLR7/8 agonists; ORN 1075 at 2.5 μg/mL or imiquimod 5 μg/mL and TLR9 agonists; C-class CpG 2429 and B-class CpG 2007 at 5 μg/mL. ORN was used with DOTAP at ORN: DOTAP ratio of 1:2. To further investigate the effect of imiquimod on CpG-induced responses, cells were co-stimulated with CpG (5 μg/mL) with variable 10-fold concentrations of imiquimod ranging from 25 μg/mL to 0.25 μg/mL. For optimal detection of cytokines, cells were stimulated for 48 hrs as previously described [200, 248]. Culture supernatants were stored at -20^oC until assayed for cytokines.

4.3.5 ELISA for IFNα, IFNγ, IL-10 and IL-12

ELISA for quantifying cytokines in cell culture supernatants were performed as previously reported for IFNα and IFNγ [203], IL-12 [214] and IL-10 [249]. Briefly, polystyrene microtiter plates (Immulon 2 for Interferons; Nunc Maxisorp for IL-12 and IL-10; Dynex Technology INC, Chantilly, USA) were coated with capture monoclonal antibodies specific for targeted cytokines. These included mouse anti-bovine recombinant IFNγ clone 2-21A [203], two mouse anti-recombinant bovine IFNα clone A2 and clone A4 [203], mouse anti-bovine IL-12 (MCA 1782EL, Serotec, North Carolina, USA) or mouse anti-recombinant bovine IL-10 clone CC318 (MCA 2110, Serotec). For every washing step, plates were washed with Tris buffer saline/0.05 % Tween 20. Cell culture supernatants and ten serial two-fold dilutions of standard concentrations of recombinant bovine IFNγ or bovine IFNα (Ciba Giegy, Basel, Switzerland), recombinant (rHuIL-12) (Serotec PHP 100) and recombinant bovine IL-10 (Kindly donated by J.C. Hope, Institute for Animal Health, Compton, UK) were then added to the plates. For IFNγ and IFNα ELISA, the captured cyokines were detected in

two steps, first by addition of rabbit anti-bovine IFNγ antisera 92-131 [211] or rabbit anti-bovine IFNα antisera 92-133 [211] followed by addition of biotinylated goat-anti rabbit IgG (Zymed, California, USA). For IL-12 and IL-10 ELISA, the captured cytokines were detected in one step by using biotinylated mouse anti-bovine IL-12 clone CC326 (Serotec MCA 2173B) or biotinylated mouse anti-bovine IL-10 clone CC320 (Serotec MCA 2111B). The reaction was developed by addition of strepavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc, USA) and visualized by using p-nitrophenyl phosphate (10 mg/mL) (Sigma-Aldrich, Ontario, Canada) as substrate.

4.3.6 Caspase assay

Caspase-3/-7 enzyme activity was determined using Caspase-Glo 3/7 assay (Promega, Madison, USA) according to manufacturer's instructions. Briefly cells were isolated as described above and stimulated with media, CpG (5 μg/mL), or CpG (5 μg/mL) + imiquimod at various concentrations (0.25, 2.5, and 25 μg/mL). After 48 hrs of incubation, 100 μL of Caspase-Glo® 3/7 reagent was added to each well of a 96-well plate. Luminescence in each sample was measured using the plate-reading Luminometer (Victor^{3}V, Perkin Elmer, CA, USA).

4.3.7 Lymphocyte Proliferative responses

Cells were re-suspended in culture medium at 2.5 x 10^5 cells per well in a final volume of 200 μL. Triplicate cultures were stimulated and incubated as already described above. During the final 6 hrs of the 72 hrs incubation, cells were pulsed with 0.4 μCi ^{3}H-Thymidine (Amersham Pharmacia, Piscataway, NJ). Cells were harvested using standard liquid scintillation protocols and uptake of ^{3}H-Thymidine was assessed in a beta counter (Topcount, Packard Instrument Company, Meriden, CT). Cell proliferation was calculated as the mean counts per minute (c.p.m) of triplicate cultures

and expressed as a stimulation index (c.p.m in the presence of stimulus/c.p.m in the absence of stimulus).

4.3.8 Quantitative RT-PCR

Total RNA was isolated from purified $CD21^+$ B cell suspensions using Trizol reagent (Invitrogen, Carlsbad, CA) and RNA samples were treated with DNAse I Amp Grade (Invitrogen) (1 U/μg of RNA). The absence of genomic DNA contamination was confirmed by use of treated RNA as template directly in PCR. RNA was quantified by determining optical density at 260 nm (OD_{260}) and the OD_{260}/OD_{280} ratio was calculated to assess purity. To obtain complementary DNA (cDNA), RNA (500 ng) was incubated in a final volume of 15 μl with dNTP (0.5 mM final each) (Invitrogen), 0.5 μg oligo dT (Invitrogen), RNAse out inhibitor (20 U) (Invitrogen), Superscript ™ reverse transcriptase (RT) (8 U) (Invitrogen), and 1× buffer RT (Invitrogen). The reaction was allowed to proceed for 10 min at 25 °C, 30 min at 42 °C and then heat inactivated at 93 °C for 5 min. The cDNA generated was either used immediately for qPCR or stored at −80 °C. The cDNA generated as above was used in the real-time PCR. cDNA was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. The primers and amplification conditions were as previously reported [250]. TLR7, 8 and 9 forward and reverse primers were synthesized from Invitrogen. The PCR conditions were 95 °C for 3 min, followed by 45 cycles with denaturation at 95 °C for 15 s, annealing temperature at 59°C for 30 s, and elongation at 72 °C for 30 s. Real-time assays were run on a Bio-Rad iCycler iQ system (Bio-Rad, Hercules, CA). The specificity of the PCR reactions was assessed by the analysis of the melting curves of the products and size verification and sequencing of the amplicons. To normalize the amount of cDNA, we sampled equal number of cells, quantified RNA, assessed its quality prior to reverse transcription, and used a reference gene. Samples were normalized internally using the average cycle threshold (Ct) of beta-actin (β actin) as a reference. Values were expressed as delta Ct value per 500 ng of total input RNA.

4.3.9 Statistical analysis

Data were analyzed using the statistical software GraphPad Prism 5 (Graphpad, San Diego, CA, USA). One-way analysis of variance (ANOVA) test was used to determine statistical differences in mean values when more than two groups were compared Values of $p<0.05$ were considered significant.

4.4 Results

4.4.1 Peyer's patches respond poorly to TLR agonists compared to LN and PBMC

Previously we have tested CpG ODN as TLR9 agonists on LN and PBMC and observed differences in responses. We wondered whether PP will respond similarly to CpG ODN as LN since both tissues are secondary lymphoid tissues. As shown in Fig. 4.1 A, mLN and PBMC responses in terms of IFNα were higher than JPP and IPP. Similarly, on stimulation with ssRNA, mLN produced higher amounts of IFNα than PBMC and PP cells (Fig. 4.1 B).

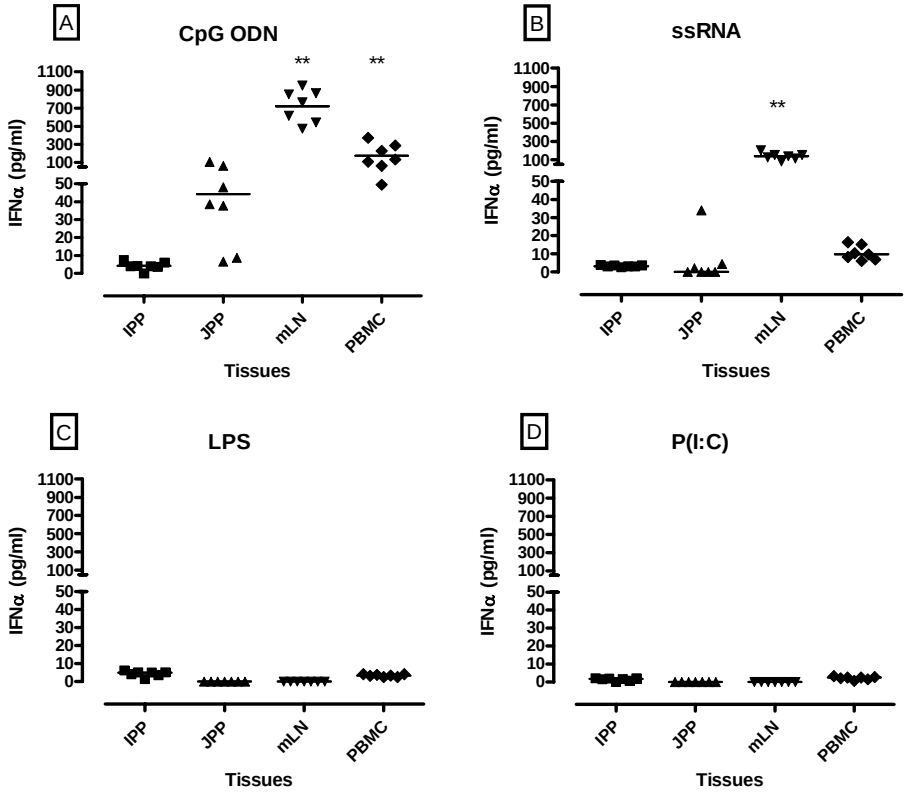

Fig. 4.1 A-D: IFNα responses in ovine IPP, JPP, mLN and PBMC following 48 hrs stimulation with TLR agonists [CpG ODN (5 µg/mL), LPS (100 ng/mL), ssRNA (2.5 µg/mL), P(I:C) (10 µg/mL)]. Data represent the mean of 7 animals (n=7). ** Significantly different from IPP ($p<0.05$).

In contrast, both LPS and P(I:C) (Fig. 4.1 C-D) did not induce IFNα from the tissues. Therefore, both CpG ODN and ssRNA induce significantly lower IFNα in JPP and IPP than in mLN and PBMC.

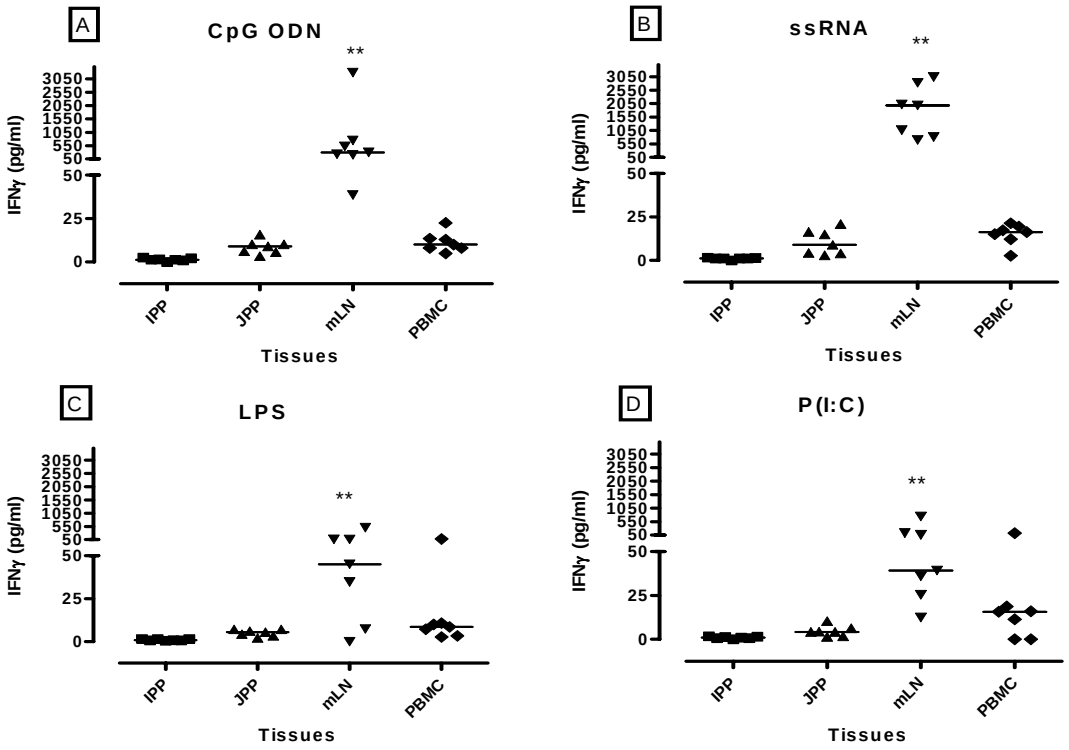

Fig. 4.2 A-D: IFNγ responses in ovine IPP, JPP, mLN and PBMC following 48 hrs stimulation with TLR agonists [CpG ODN (5 µg/mL), LPS (100 ng/mL), ssRNA (2.5 µg/mL), P(I:C) (10 µg/mL)]. Data represent the mean of 7 animals (n=7). ** Significantly different from IPP ($p<0.05$).

Consistent with previous observation, CpG ODN induced significant IFNγ from mLN but not from PBMC, JPP and IPP (Fig. 4.2 A). Similarly, on stimulation with ssRNA, mLN induced higher levels of IFNγ than PBMC, JPP and IPP (Fig. 4.2 B). This pattern of response was similar to what was observed when stimulating with CpG ODN. However, ssRNA seemed to be more potent at inducing IFNγ than CpG ODN. Similar observations were made when stimulating with LPS (Fig. 4.2 C) and P(I:C) (Fig. 4.2 D), both induced IFNγ significantly in mLN but not in PBMC, JPP and IPP. However, both

LPS and P(I:C) induced lower levels of IFNγ in mLN as compared to CpG ODN and ssRNA. These results showed that PP responses were attenuated for the TLR ligands tested as compared to mLN and PBMC.

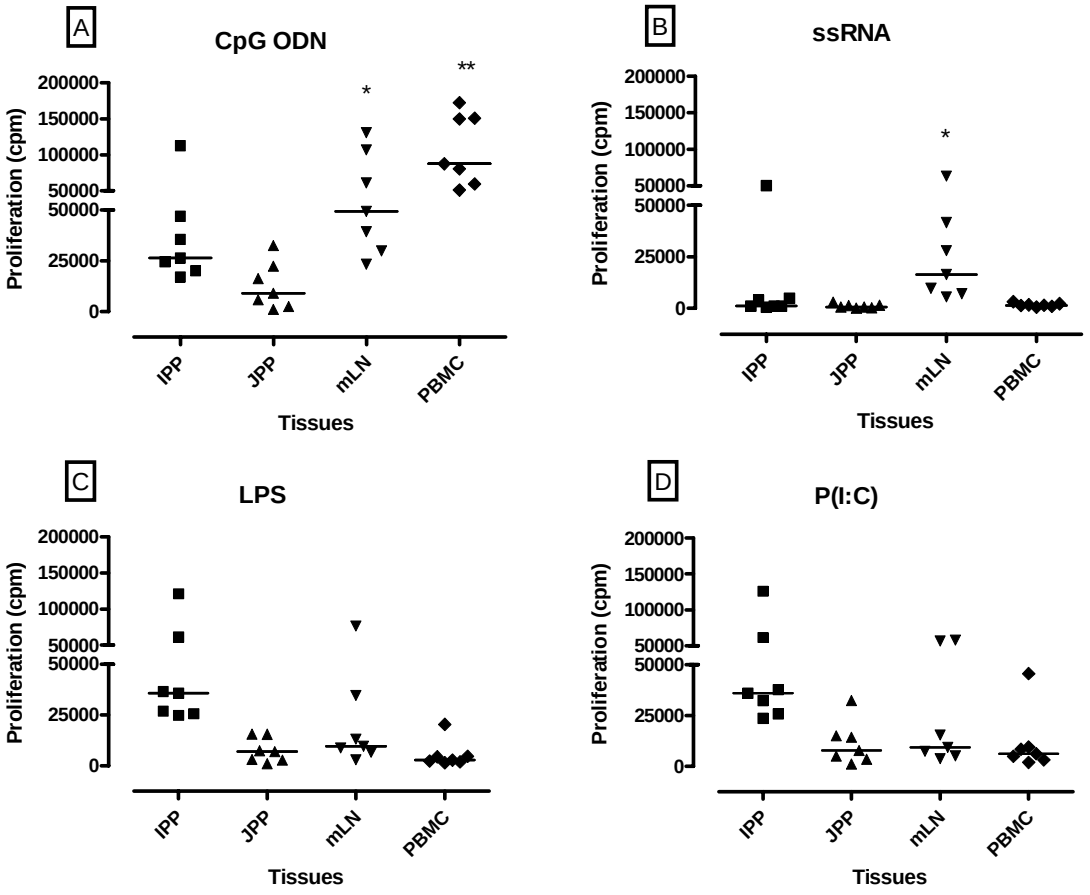

Fig. 4.3 A-D: Lymphocyte proliferative responses (c.p.m.) in ovine IPP, JPP, mLN and PBMC following 72 hrs stimulation with TLR agonists [CpG ODN (5 µg/mL), LPS (100 ng/mL), ssRNA (2.5 µg/mL), P(I:C) (10 µg/mL)]. Data represent the mean of 7 animals (n=7). ** Significantly different from IPP ($p<0.05$).

As observed previously, CpG ODN induced high level of proliferation in PBMC and mLN (Fig. 4.3 A). Upon stimulation by CpG ODN, significant proliferative responses were observed in IPP but it was reduced compared to mLN and PBMC (Fig. 4.3 A). On stimulation with ssRNA, mLN showed higher proliferative response than

PBMC and PP (Fig. 4.3 B). In contrast, no proliferation response was observed in mLN, JPP and PBMC when all tissues were stimulated with LPS (Fig. 4.3 C) or poly(I:C) (Fig. 4.3 D). IPP proliferated moderately to both agonists. LPS or P(I:C) was not a stronger inducer of proliferation than CpG ODN.

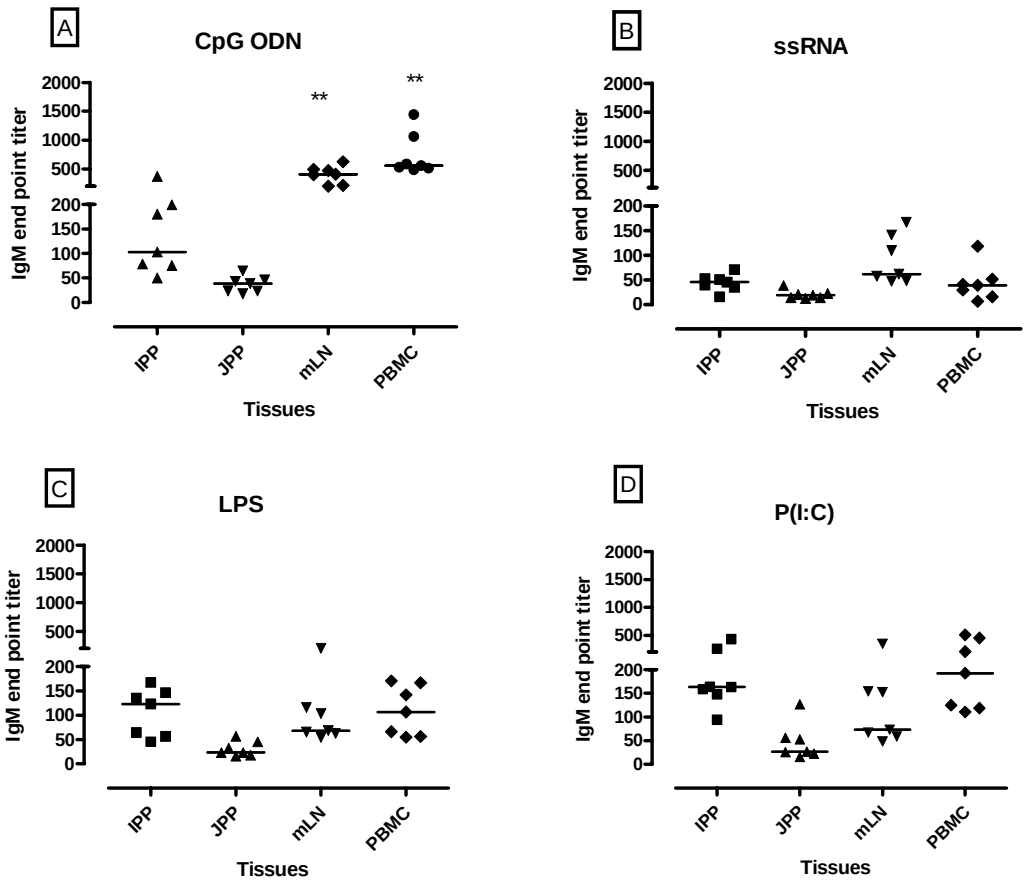

Fig. 4.4 A-D: IgM responses (end point titer) in ovine IPP, JPP, mLN and PBMC following 48 hrs stimulation with TLR agonists [CpG ODN (5 µg/mL), LPS (100 ng/mL), RNA (2.5 µg/mL), P(I:C) (10 µg/mL)]. Data represent the mean of 7 animals (n=7). ** Significantly different from IPP ($p<0.05$).

using CpG ODN and ssRNA. Interestingly IPP proliferated significantly in response to CpG ODN, LPS and P(I:C) but not to ssRNA whereas mLN cells proliferated when stimulated by CpG ODN and ssRNA but not LPS or P(I:C).

The IgM responses mirrored the lymphocyte proliferation data. PP cells secreted significantly lower levels of IgM than PBMC and mLN upon stimulation with CpG ODN (Fig. 4.4 A). Similarly, mLN produced higher level of IgM than IPP and JPP upon

stimulation by ssRNA (Fig. 4.4 B). LPS and poly(I:C) induced higher level of IgM in PBMC and mLN than in JPP (Fig. 4.4 C-D). IPP secreted high level of IgM upon stimulation with both agonists.

We wondered whether multiple stimulation of TLR agonist would trigger responses in PP cells. The lymphocyte proliferation responses were assessed on stimulation with different combinations of TLR agonists {CpG ODN (5 μg/mL), CpG + ssRNA (2.5 μg/mL), ssRNA + P(I:C) (10 μg/mL), ssRNA + LPS (100 ng/mL), P(I:C) + LPS, CpG + P(I:C)} on IPP, JPP, mLN and PBMC. As shown in Fig. 4.5 A-B, neither IPP nor JPP proliferated significantly following co-stimulation with different combinations of TLR agonists or with CpG ODN alone. PBMC and mLN proliferated significantly in response to CpG ODN stimulation (Fig. 4.5 C-D). However, stimulation with different combinations of TLR agonists did not induce cells from blood or LN to proliferate higher than with CpG ODN alone (Fig. 4.5 C-D). We noted that in PBMC and mLN, CpG-induced proliferative responses were reduced when costimulated with ssRNA. We decided to investigate this phenomenon further (See below).

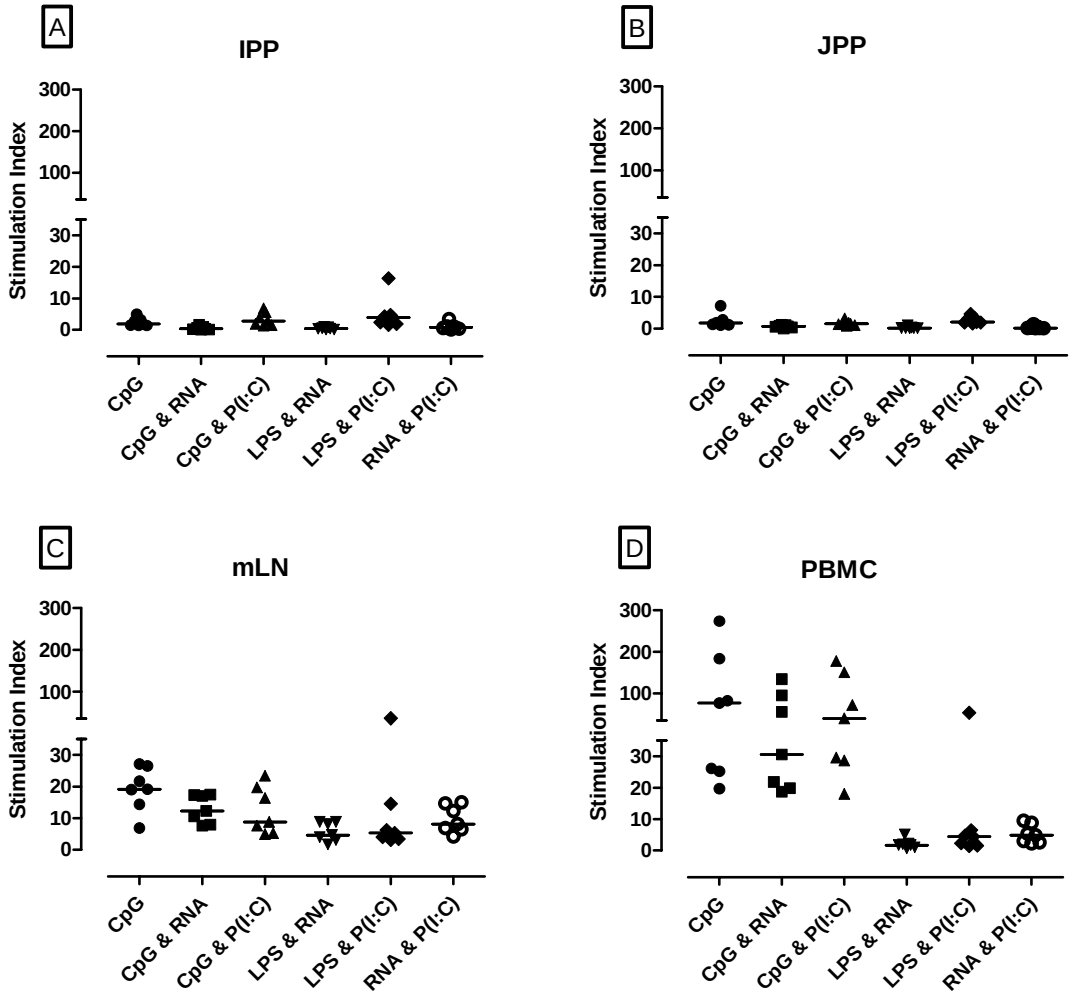

Fig. 4.5 A-D: Lymphocyte proliferative responses in ovine IPP, JPP, mLN and PBMC following 48 hrs stimulation with combination of TLR agonists [CpG ODN (5 µg/mL), LPS (100 ng/mL), RNA (2.5 µg/mL), P(I:C) (10 µg/mL)]. Data represent the mean of 7 animals (n=7).

Finally, IFNα, IFNγ, and IgM responses were assessed after stimulation with different combinations of TLR ligands {CpG ODN (5 µg/mL), CpG + ssRNA (2.5 µg/mL), ssRNA + P(I:C) (10 µg/mL), ssRNA + LPS (100 ng/mL), P(I:C) + LPS, CpG + P(I:C)} on IPP, JPP, mLN and PBMC. The results showed that there was no synergistic responses for the combinations of agonists tested in IPP, JPP and PBMC for all the responses tested (Data not shown). Thus PP responses were attenuated compared to the lymph node even when stimulated with multiple TLR ligands.

4.4.2 PP express similar levels of TLR mRNA to mLN and PBMC

Since TLR-induced responses were poor in PP, we wondered whether TLRs were expressed in sheep PP cells. Therefore the expression of TLR7, TLR8 and TLR9 was determined in IPP, JPP, mLN and PBMC using real time PCR. As shown in Fig. 4.6 A, the levels of TLR7 mRNA expression were similar in mLN, PP and PBMC. Similarly, the levels of TLR8 mRNA expression are similar in all tissues tested (Fig. 4.6 B). Therefore the poor responses of TLR7/8 agonist were not due to a lack of TLR receptors.

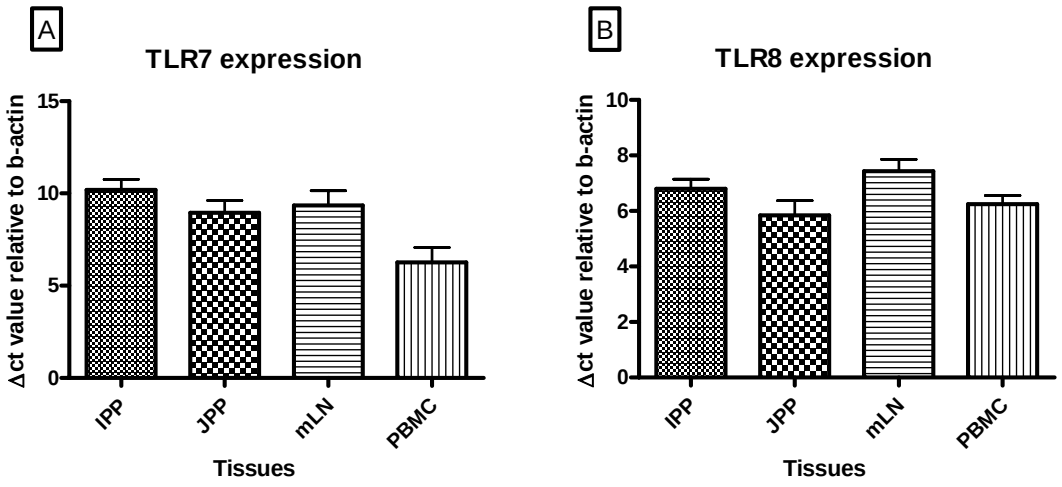

Fig. 4.6 A-B**:** Expression of TLR7 and TLR8 in IPP, JPP, mLN and PBMC. Data represent the mean of 7 animals (n=7).

The level of TLR9 expression was also assessed in IPP, JPP, mLN and PBMC. As shown by Fig. 4.7, IPP, mLN and PBMC expressed equal amount of TLR9 copy number whereas the level of copy transcript in JPP was lower but not statistically different from the other tissues. Therefore the poor responses of PP cells to CpG ODN stimulation were not due to a lack of TLR9 receptor.

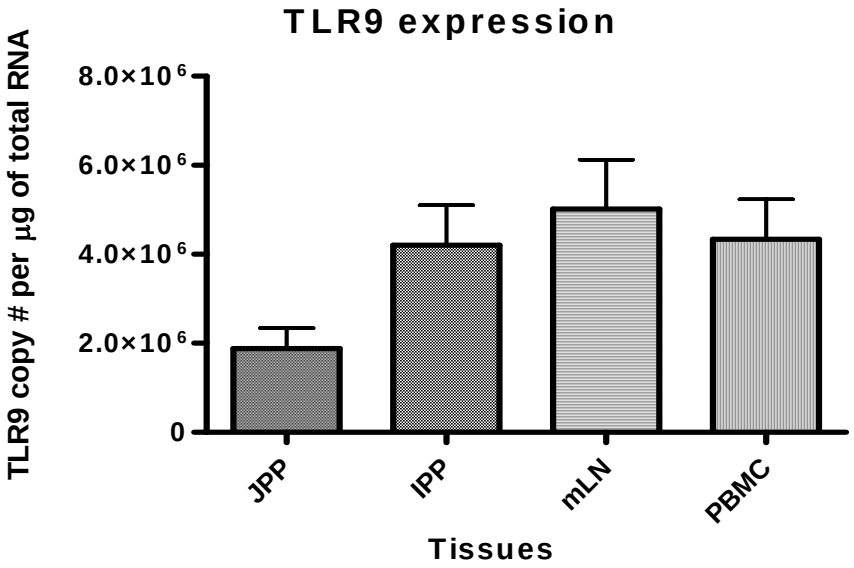

Fig. 4.7: Expression of TLR9 in IPP, JPP, mLN and PBMC. Data represent the mean of 7 animals (n=7).

4.4.3 Oligoribonucleotides suppress the CpG-induced innate immune responses in peripheral blood, mesenteric lymph nodes and purified B cells.

Since both ORN and CpG induce innate immune responses, we wondered whether simultaneous activation with both ORN and CpG will lead to additive or synergistic responses. However, results showed that co-stimulations with the two TLR agonists had a negative rather than a positive effect on the resulting innate immune responses. Compared to responses induced by CpG alone, the IFNα responses induced by ORN+CpG was lower ($P<0.01$) in PBMC (Fig. 4.8 A) as well as in mLN ($p<0.001$) (Fig. 4.8 B).

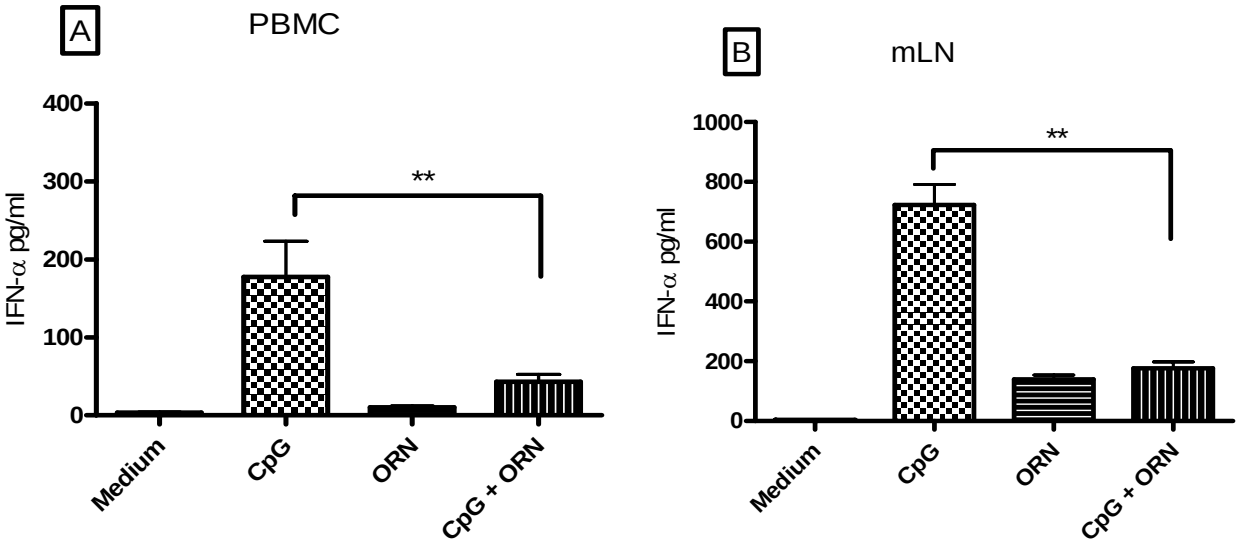

Fig. 4.8 A-B: Simultaneous cell activation with RNA oligonucleotides (ORN) and CpG ODN reduce the CpG-induced IFNα response. Ovine mononuclear cells from peripheral blood (PBMC) (A) and mesenteric lymph nodes (mLN) (B) collected from 7 animals were stimulated with 5 μg/mL CpG 2429 alone, 2.5 μg/mL ORN 1075 alone, or both for 48 hours and IFNα responses measured by ELISA. Significant difference between CpG alone and CpG + ORN (**P<0.05).

The suppression of CpG-induced responses by ORN was also reflected at the level of IgM responses as well. CpG alone significantly ($p<0.001$) induced IgM responses in PBMC (Fig. 4.9 A) and mLN (Fig. 4.9 B). But CpG+ORN caused a significant reduction in IgM ($p<0.05$) responses in PBMC (Fig. 4.9 A). The IgM response in mLN (Fig. 4.9 B) was also reduced by CpG+ORN though not significantly.

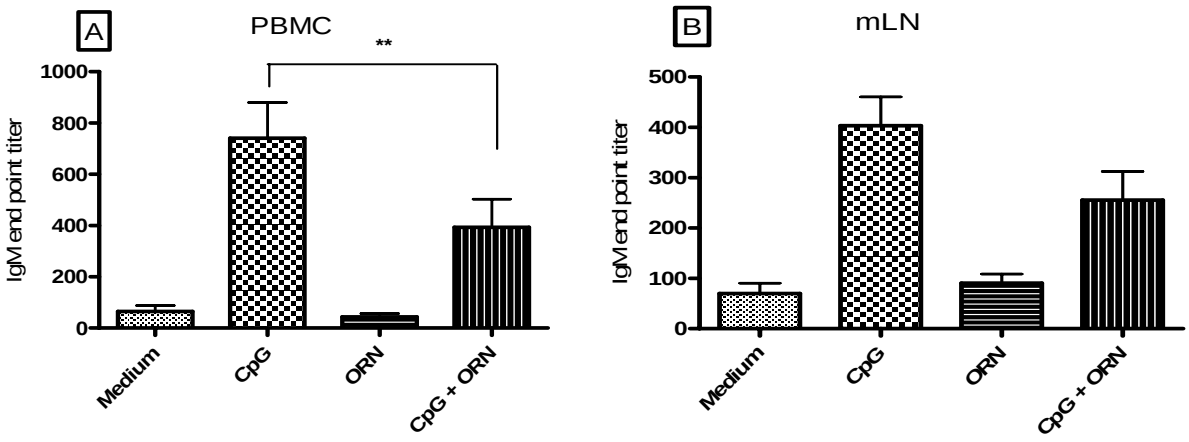

Fig. 4.9 A-B: ORN suppress the CpG-induced IgM response in cells simultaneously activated with the two TLR agonists. Ovine mononuclear cells from peripheral blood (PBMC) (A) and mesenteric lymph nodes (mLN) (B) from 7 animals were stimulated with 5 µg/mL CpG 2429 alone, 2.5 µg/mL ORN 1075 alone, or both for 48 hrs and IgM responses were measured by ELISA. Significant difference between CpG alone and CpG + ORN (**P<0.05).

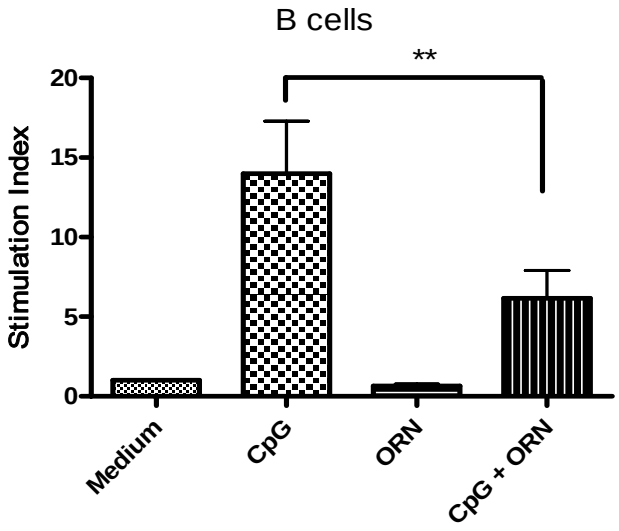

Fig. 4.10: Suppression of CpG-induced proliferative responses in purified ovine B cells following co-stimulation with RNA oligoribonucleotides (ORN). $CD21^+$ B cells were purified from peripheral blood of 7 animals by magnetic activated cell sorting (MACS). The cells were stimulated with 5 µg/mL CpG 2429 alone, 2.5 µg/mL ORN 1075 alone, or both for 72 hours and the proliferative responses measured based on ^{3}H-thymidine incorporation. Significant difference between CpG alone and CpG + ORN (**P<0.05).

The suppression of CpG-induced responses by ORN was also observed at the level of purified B cells proliferative responses. CpG induced significant B cells proliferation ($p<0.001$) which was significantly reduced ($p<0.01$) following co-stimulation with ORN (Fig. 4.10).

4.4.4 Imiquimod, another TLR7/8 agonist also suppresses CpG-induced responses in a dose dependent manner

To confirm that the downregulation of CpG-induced responses was specific to the receptor, and not an artifact of the ORN molecule, we co-stimulated cells with CpG along with imiquimod (a synthetic small antiviral compound and a known TLR7/8 agonist) at increasing ten-fold concentrations ranging from 0.25 μg/mL to 25 μg/mL.

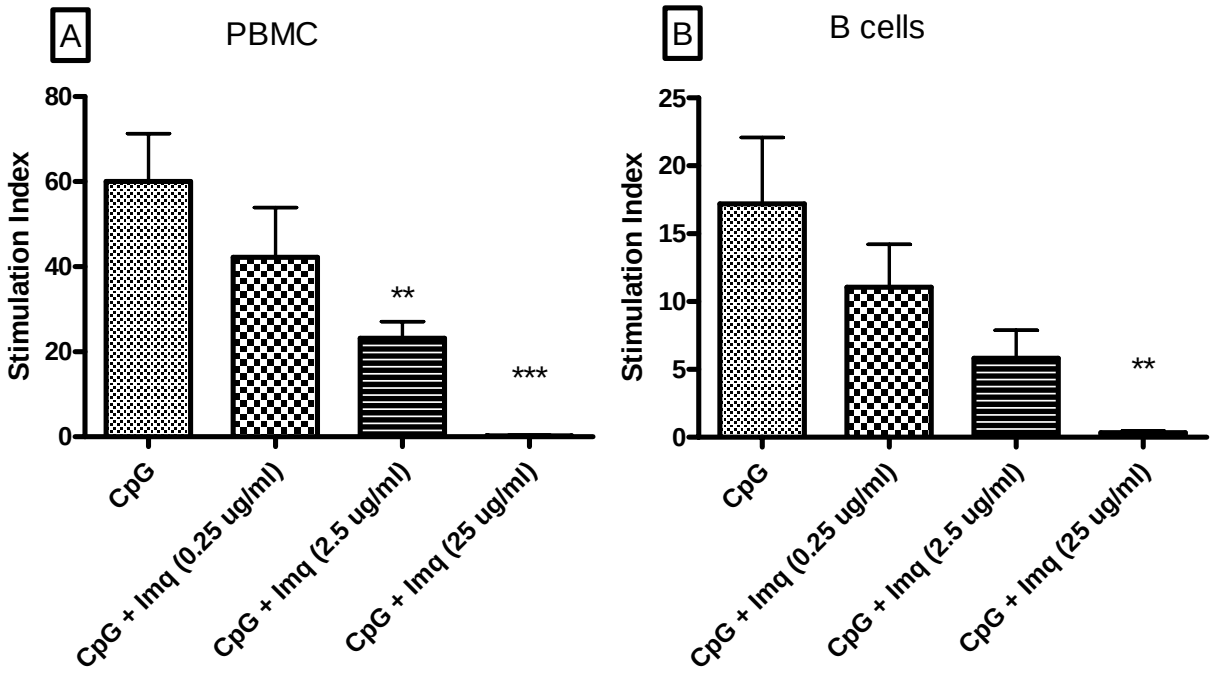

Fig. 4.11 A-B: The RNA oligoribonucleotides (ORN)-induced suppression of CpG-induced proliferative responses is dose-dependent. Ovine PBMC (Fig. 17A) as well as purified $CD21^+$ B cells (Fig. 17B) from 7 animals were stimulated with CpG along with increasing 10-fold concentrations of imiquimod (Imq) and the proliferative responses measured based on ^{3}H-thymidine incorporation. Data is presented as proliferation relative to un-stimulated cells (stimulation index). Significant difference between treatments (**$p<0.05$, ***$p<0.001$).

The CpG-induced PBMC proliferative responses decreased progressively with increasing imiquimod concentrations and was significantly lower at imiquimod concentrations of 2.5 μg/mL ($p<0.05$) and 25 μg/mL ($p<0.001$) (Fig. 4.11 A). Therefore the antagonistic effect to CpG responses is not specific to the nature of the TLR7/8 agonist used but probably specific to the receptor (TLR7/8 activation). A similar dose-depended suppression of CpG-induced proliferation by imiquimod was seen for purified B cells (Fig. 4.11 B).

4.4.5 Reduction of CpG-induced responses by TLR7/8 agonists is not associated with induction of cell death or the anti-inflammatory cytokine IL-10

From this point, all subsequent experiments were done with imiquimod which is more convenient to handle (ORN requires prior conjugation to a carrier molecule, DOTAP, which enhances its stability and increases its uptake by cells).

We then evaluated whether the inhibitory effects of the TLR7/8 and TLR9 costimulation was due to production of the immunoregulatory molecule IL-10 or induction of programmed cell death (apoptosis). We measured the levels of the pro-apoptotic caspase enzymes in the co-stimulated B-cells as well as the levels of the immunosuppressive IL-10 in culture supernatants of stimulated cells.

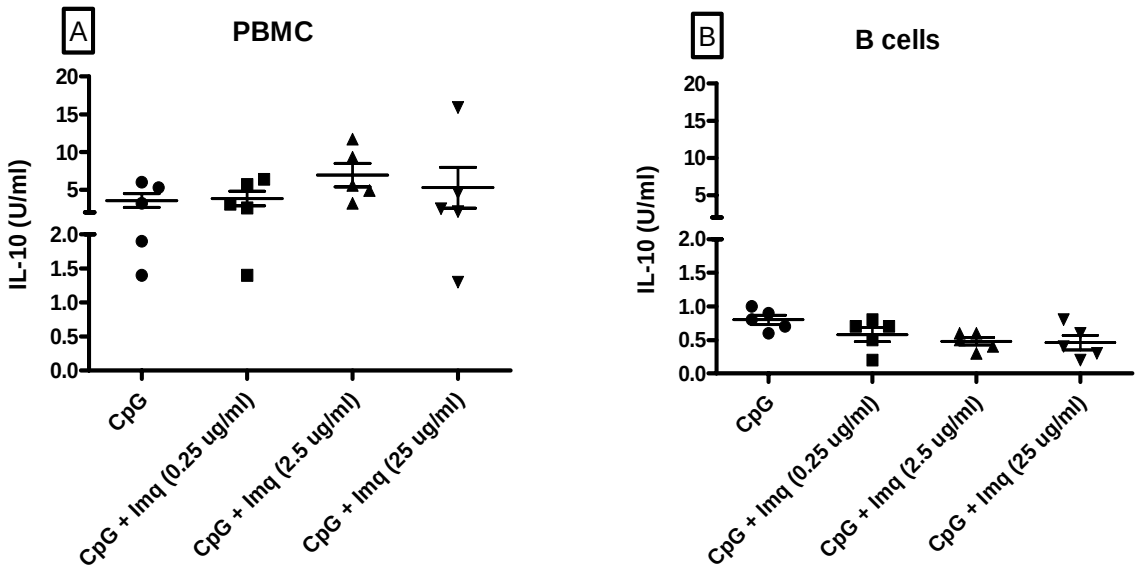

Fig. 4.12 A-B: IL-10 responses by ovine peripheral blood mononuclear cells (PBMC) and purified B cells. PBMC and isolated B cells from 5 animals were stimulated with 5 µg/mL CpG 2429 or CpG 2429 + imiquimod (0.25, 2.5 and 25 µg/mL).

There was however no difference in pro-caspase enzyme activity or IL-10 levels between cells stimulated with CpG alone and those simultaneously activated with CpG plus with increasing imiquimod concentrations indicating that the reduced responses were not due to inhibition by IL-10 (Fig. 4.12 A-B) or induction of activation-induced cell death (Fig. 4.13).

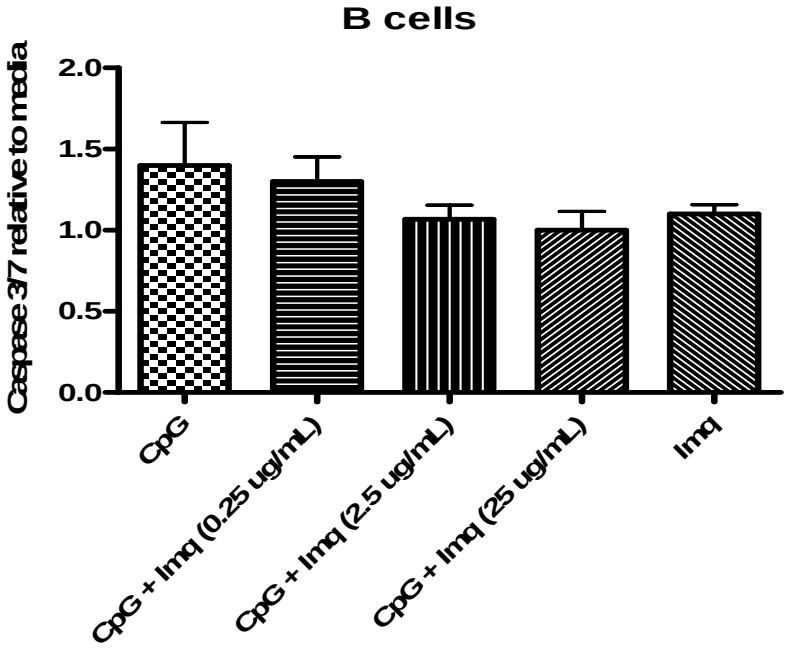

Fig. 4.13: Caspase 3/7 responses by ovine purified PBMC B cells stimulated with 5 µg/mL CpG2429 or CpG 2429 + imiquimod (0.25, 2.5 and 25 µg/mL) or imiquimod (Imq) (5 µg/mL) for 48hrs. Number of animals used is four (n=4).

4.4.6 Suppression of CpG-induced responses was not due to preferential cellular uptake of the TLR7/TLR8 agonists

Since imiquimod is a rather small molecule, we wondered whether it was preferentially taken up by cells and therefore engage the receptors and/or the downstream signaling intermediates at the expense of CpG. We therefore monitored the responses when cells were first exposed to CpG for variable time periods before addition of imiquimod. We used two different CpG sequences; B-class CpG 2007 and C-class CpG 2429. Surprisingly, addition of imiquimod 2 hours or even 4 hours after CpG treatment still resulted in a significant reduction ($p<0.05$) of the CpG-induced responses in PBMC (Fig. 4.14 A) indicating that preferential uptake of imiquimod cannot explain the reduction of the CpG-induced responses. This suppression occurred when B-class CpG 2007 or when C-class CpG 2429 were used indicating that it is not associated with CpG class or sequence. Similarly, addition of TLR7/8 agonists to

purified B-cells 2 or 4 hours after stimulation with CpG suppressed the CpG-induced proliferation (Fig. 4.14 B).

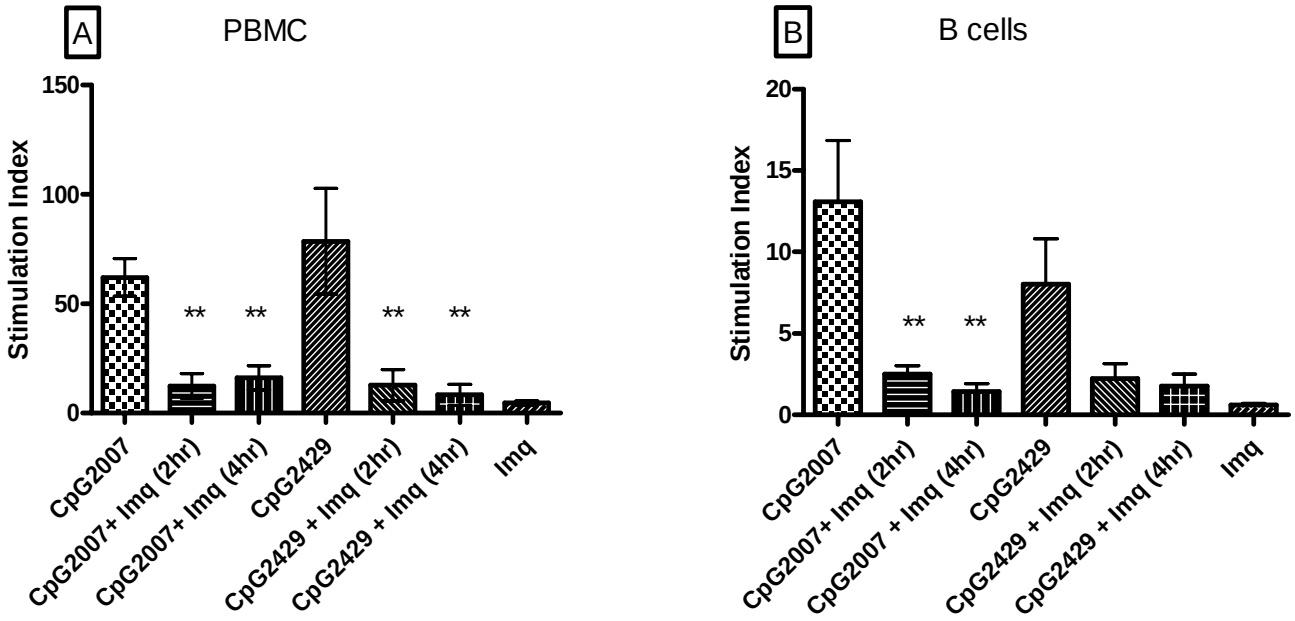

Fig. 4.14 A-B: Proliferative responses by ovine peripheral blood mononuclear cells (PBMC) (A) and purified B cells (B). PBMC and $CD21^+$ B cells from 7 animals were stimulated with 5 µg/mL B-class CpG2007 or C-class CpG 2429 for 2 or 4 hours before addition of 5 µg/mL imiquimod (Imq). Proliferative responses were measured by ^{3}H-thymidine incorporation. **Significant difference between treatments (**$p<0.05$).

4.5 Discussion

In the present study, we investigated innate immune responses induced by TLR agonists or their combinations in immune cells from sheep. The results demonstrated for the first time in ovine species that the PP immune responses were poor compared to LN and PBMC following stimulation with various TLR agonists despite the expression of TLR mRNA. Moreover, multiple stimulations with combinations of TLR agonists did not show any significant increase in responses in PP. Interestingly, we observed that TLR7/8 agonists in combination with CpG ODN downregulated the CpG-induced IFNα, proliferation and IgM responses in PBMC as well as proliferative responses in purified B cells.

The responses in terms of proliferation following CpG stimulation represent mainly B cell responses in the tissues tested. Our results showed that PP responses in terms of proliferation were significantly less compared to PBMC. These proliferation responses were quite surprising as IPP and JPP consist of more than 90% and 70% of B cells, respectively, whereas in PBMC, the percent B cells composition varies around 30-40%. These results suggested that in PP, either the expression of TLR9 was downregulated or the cells were regulated differently. Given that the gut is always exposed to PAMPs from commensals, the PP may have evolved mechanisms to dampen the immune responses.

The expression of TLR9 was determined using real-time RT-PCR and our results demonstrated that there was expression of TLR9 mRNA in both IPP and JPP comparable to the TLR9 expression in the LN and PBMC. These expression profiles of the PP and mLN correlate with the TLR profiles reported recently for sheep tissues [251, 252]. However none of those reports investigated further into the functional properties of the PP and mLN. Therefore our data demonstrated that PP innate responses to CpG ODN are poor despite expression of TLR9.

Similarly, other TLR agonists such as poly(I:C), LPS, ssRNA did not induce any significant responses in PP cells. These results suggest that the poor responses of PP are not an artifact of CpG ODN or of the TLR9 receptor. Rather it seems that the PP cells

have evolved regulatory mechanisms to "ignore" TLR agonists and in this regard they respond differently from other sterile immune tissues such as blood and LN. There are similar studies that suggest that mucosal cells such as intestinal epithelial cells (IEC) or macrophages have adapted to their environment and do not respond directly to TLR stimulation [126, 127, 134]. It is worth noting that upon simultaneous stimulation of multiple TLR agonists, PP responses were still significantly less compared to PBMC and LN and no synergistic responses were observed.

However in PBMC and mLN, we observed that the CpG-induced immune responses were inhibited upon simultaneous stimulation with multiple TLR agonists. Co-stimulation of PBMC with TLR7/8 and TLR9 results in reduction in CpG-induced IFNα responses and this is in agreement with previous reports using human PBMC or pDC [241, 242, 253]. However, our results differ with those of Marshall and colleagues [242] who reported that co-stimulation with these agonists did not have any effect on human B cells proliferation and IL-6 production. We observed that cell proliferation and IgM production were reduced in ovine B cells following co-stimulation of TLR7/8 and TLR9, but we were not able to assess IL-6 response by ELISA because reagents were not available. The reasons for the difference between responses to TLR co-stimulation in human and ovine B cells are not known but may involve cross-talk between the receptors. It was suggested that TLR7 agonists antagonized CpG through intracellular mechanisms because TLR7 and TLR9 are present on pDC [253]. Intracellular interactions or cross-talk between TLR7/8 and CpG mediated signaling may explain the down-regulation of B cell responses observed in this study because sheep B cells expressed TLR7, TLR8 and TLR9 mRNA. Absence of cross-talk between TLR7 and TLR9 may explain the failure of the TLR7 agonist to antagonize CpG in human B cells [242] probably because naïve human B cells do not co-express TLR7 and TLR9. Further, we demonstrated the same effect in mLN and thus it was not tissue specific.

By using a highly purified B cell population we were able to perform mechanistic studies which would otherwise be difficult to conduct in mixed cell population like PBMC. Two issues are worthy of consideration when co-stimulating cells with CpG and imiquimod. The signaling pathways for TLR7/8 and TLR9 are similar in that they are both dependent on the adaptor molecule MyD88 and this may

lead to competition between the two agonists for downstream signaling intermediates. Secondly, because of its smaller size, imiquimod may be taken up more rapidly and engage the downstream signaling intermediate at the expense of CpG [241]. However, our results show that kinetic cellular uptake advantage or competition does not explain the antagonism mediated by TLR7/8 agonists because the suppression was not only observed when the TLR7/8 and CpG agonists were added simultaneously but also when cells were sequentially stimulated with CpG for 2-4 hours prior to stimulation with the TLR7/8 agonist. CpG is internalized by cells and engage TLR9 within 30 minutes [254, 255]. By 4 hours after exposure, CpG will most likely have initiated downstream signaling cascades before the TLR7/8 agonist was introduced.

The interest in multiple TLR stimulation arises from the fact that pathogens express multiple PRRs and therefore simultaneous or sequential stimulation of more than one TLR (and non-TLR) is highly likely in vivo [241, 242, 253, 256]. Also, co-infection with more than one pathogen would increase the likelihood of multiple TLR stimulation. It is thought that two complementary TLR agonists may induce stronger immune responses and better protection than a single TLR agonist. The requirement for multiple signals to induce potent immune responses has been proposed as a mechanism by which the immune system exerts a stringent "combinatorial security code" whereby at least two microbial products are required to stimulate a strong immune response upon encounter with a pathogen [94]. Such synergistic effects would result in robust immune responses and lead to better protection against infection. For example, using double gene knockout mice (TLR2-/- TLR9-/-), it was shown that TLR2 and TLR9 cooperate in the control of pathogen replication following *Mycobacterium tuberculosis* and *Trypanosoma cruzi* infections [257, 258]. Detailed information regarding combined TLR stimulation may have implication in the development of more effective vaccines.

In this regard, it was recently reported that attenuated live yellow fever vaccine, one of the most successful vaccines, simultaneously and potently activates TLR2, TLR7, TLR8 and TLR9 on different DC subsets [259]. This may explain why single TLR agonist have not been as successful adjuvants or immunotherapeutics in large animals and humans as previously expected [260]. While cooperation among TLR during infection may result in more robust immune responses and protection, these

responses must be downregulated to maintain tissue homeostasis, thereby avoiding immunopathology. In this regard, inhibitory effects at the appropriate time would be necessary to prevent uncontrolled responses which would otherwise lead to tissue injury. Therefore, TLR7/8 signals on top of TLR9 signaling activate down-regulatory mechanisms to avoid potential pathological consequences of overstimulation of immune responses as suggested by others [261, 262].

It is not fully understood what exactly determines synergy or inhibition/tolerance between TLR agonist combinations. Bagchi et al [95] have recently suggested that the outcome may be influenced by the differential requirements of the MyD88-dependent (D) and MyD88-independent (I) intracellular signaling pathways. By studying agonists in various categories, these investigators found that simultaneous or sequential activation of both D and I pathways causes synergistic or complementary responses, while tolerance or inhibition is caused by agonists that act through the same pathways [95]. Our results are consistent with this pattern, since TLR7/8 and TLR9 all utilize the D pathway.

4.6 Conclusion

We have shown that PP respond poorly to TLR agonists despite expressing high levels of TLR mRNA. Simultaneous stimulation of TLR agonists was not sufficient to elicit innate responses in PP tissues. Moreover we have shown in this study that synthetic and natural TLR7/8 agonists activate innate immune responses in sheep which was not reported previously. Furthermore, we show that simultaneous stimulation of sheep cells with CpG and the TLR7/8 agonists resulted in suppression of responses in blood mononuclear cells including purified B cells.

CHAPTER 5: A NOVEL REGULATORY B CELL POPULATION IN SHEEP PEYER'S PATCHES SPONTANEOUSLY SECRETES IL-10 AND DOWNREGULATES TLR-9-INDUCED IFNα RESPONSES.

5.1 Abstract

Peyer's patches (PP) play an important role in the induction of immune responses in the intestine, but regulation of TLR-induced innate immune responses in PP is not well understood. We investigated the responses of PP and other immune cells to the TLR9 agonist, CpG ODN. PBMC and LN cells secreted significant amounts of interferon (IFNα, IFNγ), and interleukin (IL)-12 following stimulation with CpG ODN. In contrast, PP cells exhibited poor cytokine responses, despite abundant expression of TLR9 mRNA. PP cells spontaneously secreted high levels of IL-10, and the primary source of the IL-10 was $CD5^{-}CD11c^{-}CD21^{+}$ B cells. Neutralization of the IL-10 or depletion of $CD21^{+}$ B cells resulted in a significant increase in CpG-induced IFNα response in PP, suggesting that IL-10 from $CD21^{+}$ B cells regulate innate responses in PP. These IL-10-secreting PP $CD21^{+}$ B cells may represent a novel subset of the recently proposed regulatory B cells (B_{regs}) in the intestine.

5.2 Introduction

The innate immune system has an intricate system of receptors called pattern recognition receptors (PRR) which have the ability to recognize pathogen-associated molecular patterns (PAMPs) as danger signals [4, 18]. On recognition of their respective ligands, PRR are capable of inducing a variety of innate immune responses. The TLR constitute one such family of PRR and are evolutionarily conserved transmembrane glycoproteins molecules [167, 170]. At least thirteen TLR have been identified in mammals and they appear to detect distinct microbial components. For example, TLR3, TLR4, TLR7/8 and TLR9 recognize double-stranded RNA, lipopolysaccharides (LPS), single strand RNA/imidazoquinolines and CpG motifs in bacterial DNA, respectively [41, 171-174]. In bacterial DNA, CpG dinucleotides occur in the expected frequency (1/16) while in vertebrate DNA, this frequency is reduced (1/64). The cytosine in CpG dinucleotides is often methylated in vertebrate DNA [175]. Thus, the vertebrate immune system has evolved to specifically recognize CpG dinucleotides as a way of detecting the presence of microbial organisms.

Both *in vitro* and *in vivo* studies have demonstrated that CpG ODN are potent activators of the innate immune system in numerous species including humans, non-human primates, mice, cattle, sheep, pigs, horses, dogs, cats, chickens and fish [53, 56, 176-181]. However, CpG-specific responses differ from species to species and the differences in responses seem to be related to TLR9 expression in different cell types. For example, in resting human cells, B cells and plasmacytoid dendritic cells (pDC) are the two cell types that predominantly express TLR9 and are directly activated by CpG ODN [43, 92]. In contrast, in mice, a wide variety of cell types express TLR9 and are activated directly by CpG ODN including B lymphocytes, monocytes, macrophages, myeloid dendritic cells, NK cells, T-regulatory cells, intestinal and pulmonary epithelia and even mast cells [62, 103, 263-265]. All of these cell types express functional TLR9 and this explains why CpG ODN are so potent in mice. TLR9 expression in ruminants has been recently reported [93, 251] and appears to have an expression pattern similar to that of humans [93]. We recently reported that sheep lymph node cells respond well to all three classes of CpG ODN and with higher magnitude than PBMC [203, 238].

However, responses in gut-associated lymphoid tissues (GALT) to TLR9 ligand stimulation have not been explored in detail.

The intestinal tract is constantly exposed to microbial flora and must have mechanisms in place to prevent unnecessary and potentially damaging immune responses to harmless commensal flora and dietary antigens. Several mechanisms have been proposed including downregulation of TLR receptor expression in lamina propria macrophages [126], lack of innate receptor expression (CD14, Fcα, Fcγ, CR3 and CR4) in intestinal macrophages [127], restricted distribution of TLR expression in intestinal epithelial cells [86, 266, 267] and modulation of intestinal epithelia cells by commensal microflora [268]. However whether such regulation of immune responses occurs in PP is not fully understood.

PP are the primary site where adaptive immune responses are induced in the intestine of ruminants [110]. Given the role of this tissue in the initiation of immune responses and the importance of TLR in the initiation and development of innate and adaptive immune responses, a detailed understanding of how PP cells respond to TLR agonists would provide insights into the regulation of immune responses in intestine.

Contractor *et al* recently reported that murine PP pDCs, unlike splenic pDCs, were incapable of producing interferon (IFNα) following stimulation with CpG ODN, presumably due to the suppressive effects of interleukin (IL)-10 in the PP environment [143]. Recently, IL-10-secreting B cells with a regulatory role have been described in murine models of chronic intestinal inflammation [152]. We report that PP $CD21^{+}$B cells isolated from normal intestinal tissue spontaneously secrete IL-10, which in turn suppresses CpG-induced innate responses.

5.3 Materials and methods

5.3.1 Oligodeoxynucleotides

A-class (2216), B-class (2007) and C-class (2429) CpG ODN have previously been shown to be biologically active in sheep PBMC and LN in vitro and in vivo [210, 211, 238]. Table 3.1 shows the sequences of the six ODNs used in these studies (See section 3.3.1). CpG ODN sequences 2007, 2216 and 2336 were obtained from Merial Limited (Lyon, France), while CpG ODN sequence 2429 were provided by Coley Pharmaceutical group (Wellesley, MA, USA) and control non-CpG ODNs 2007GC and 2216GC were purchased from Operon (Alameda, CA, USA).

5.3.2 Animals

Suffolk sheep of either sex (3 to 4 months of age) were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). The animals were housed at the Vaccine and Infectious Disease Organization (VIDO) animal facility and fed ad libitum on a ration of rolled barley and alfalfa hay. All experiments were carried out according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Experimental protocols were approved by the University of Saskatchewan Animal Care Committee and animals were housed in the same pen throughout each experiment.

5.3.3 Isolation of PBMC, JPP, IPP and LN cells

Blood was collected from the jugular vein of sheep in ethylene diamine tetraacetic acid (EDTA)-treated vacutainer tubes (BD Biosciences, Mountain View, CA, USA) and PBMC were isolated using 54% isotonic Percoll^{TM} (Pharmacia Biotech AB, Uppsala, Sweden), as described previously [201, 212]. Cells were counted using a cell counter (Dual Diluter III, Coulter electronics Ltd, Luton, England) and resuspended in

Aim-V medium (GibcoBRL, Burlington, ON, Canada) containing 2% fetal bovine serum (FBS; GibcoBRL).

Sheep were euthanised and scLN and mLN were removed and placed in ice-cold minimum essential medium (MEM, GibcoBRL) containing the antibiotics 100 IU/mL Penicillin, 100 μg/mL Streptomycin sulfate and 0.25 μg/mL Amphotericin B (Sigma-Aldrich, St Louis, Missouri, USA). Cells were isolated from LN by finely mincing tissue with a scalpel, filtering the cell suspension through a 40-μm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and washing cells with Dulbecco's Phosphate Buffered Saline (PBSA) (pH 7.2), as described previously [213]. JPP and IPP tissues were also removed and placed in MEM (GibcoBRL) containing antibiotics (100 IU/mL Penicillin, 100 μg/mL Streptomycin sulfate, 0.25 μg/mL Amphotericin B) (Sigma-Aldrich). The PP cells were also isolated from the same sheep as described previously [110, 245]. The number of viable cells in all tissues was determined by trypan blue dye exclusion and counting with a hemocytometer under a light microscope. Cells were resuspended in AIM V medium containing 2% FBS.

5.3.4 Tissue culture conditions

Stimulation of PBMC, JPP, IPP and LN cells was performed in 96-well, round bottom plates (Nunc, Naperville, IL, USA) using AIM V medium supplemented with 2% FBS, 100 IU/mL Penicillin, 100 μg/mL Streptomycin sulfate, 0.25 μg/mL Amphotericin B, 2mM L-glutamine, 50μM 2-Mercaptoethanol and 10μg/mL Polymyxin B Sulfate (Sigma-Aldrich). For the dose titration experiment, PBMC and LN were incubated with either 0.66, 2.0, 6.0 or 18.0 μg/mL of each type of CpG ODN (2336, 2007, 2429, 2007GC) for 48 hrs at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Forty eight hours was previously shown to be optimal time for detection of cytokine secretion in ovine PBMC stimulated with CpG ODN [200]. For each treatment, $5x10^5$ cells were cultured in triplicate wells in 200 μL total volume. Culture supernatants were harvested and stored at –20°C until assayed for IFNα, IFNγ.

5.3.5 Enzyme-linked immunosorbent assay (ELISA) for IFNα, IFNγ, IL-10 and IL-12

The IFNα, IFNγ, IL-10 and IL-12 ELISA assays used during this study were previously shown to detect ovine and bovine IFNα, IFNγ and IL-12 cytokines as described elsewhere [210, 213, 214, 249]. Briefly, polystyrene microtiter plates (Immulon 2; Dynex Technology Inc, Chantilly, USA) were coated with capture antibody. For IFNγ ELISA, mouse anti-bovine IFNγ antibody (clone 2-2-1) [213] and for IFNα ELISA, two mouse anti-bovine IFNα antibodies (clones IFN-1C6 and IFN-1D10) were diluted in coating buffer [215]. For IL-12, mouse anti-recombinant bovine IL-12 antibodies (Serotec MCA 1782EL, NC, USA) were diluted to 8 μg/mL in coating buffer. For IL-10, mouse anti-bovine IL-10 antibodies (Serotec MCA 2110, NC, USA) were diluted to 0.5 μg/mL in coating buffer and applied to Maxisorp 96U plates (Dynex Technology Inc, Chantilly, USA). Plates were washed with Tris buffer saline/0.05 % Tween 20 (Sigma-Aldrich) (TBST) at all steps. Recombinant bovine IFNγ (bIFN-γ), bovine IFNα (bIFN-α) (Ciba Giegy) and recombinant human IL-12 (rHuIL-12) (Serotec PHP 100) and recombinant bovine IL-10 (Kindly donated by J.C. Hope, Institute for Animal Health, Compton, UK) were used as standards. Standards and samples were diluted in TBST/0.5% gelatin (Sigma-Aldrich) (TBST-g) and added to the wells. For IFNγ and IFNα ELISA, the captured cyokines were detected in two steps, first by addition of rabbit anti-bovine IFNγ antisera 92-131 [203] or rabbit anti-bovine IFNα antisera 92-133 [211] followed by addition of biotinylated goat-anti rabbit IgG (Zymed, California, USA). For IL-12 and IL-10 ELISA, the captured cytokines were detected in one step by using biotinylated mouse anti-bovine IL-12 clone CC326 (Serotec MCA 2173B) or biotinylated mouse anti-bovine IL-10 clone CC320 (Serotec MCA 2111B). Strepavidin-alkaline phosphatase (Jackson ImmunoResearch, Westgrove, PA) were used for detection. The assay was developed by using p-nitrophenyl phosphate (Sigma-Aldrich) substrate in 1 % diethanolamine (Sigma-Aldrich) and 0.5 mg/mL magnesium chloride. The reaction was stopped by adding 0.3M EDTA to each well. Optical density of the reaction product was measured at 405 nm using a 490 nm reference on a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Sample

concentrations were calculated using Microplate Manager 5.0.1 version software (Bio-Rad).

5.3.6 IFNα ELISPOT Assay and neutralization of IL-10

IFNα-secreting cells (ISC) were detected using an ELISPOT assay. Microtiter nitrocellulose filtration plates (Whatman, Clifton, NJ) were coated overnight with two monoclonal mouse anti-bovine IFNα antibodies (clones IFN-1C6 and IFN-1D10) and were both diluted to 1:1000. Wells were washed with RPMI complete media to remove free protein before adding 1×10^6 cells/well to triplicate wells, in a final volume of 200 µl culture medium. Cultures were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 before removing the cells. Total ISC were detected by first adding biotinylated goat anti-rabbit IgG (H+L chain specific; 1:5000 dilution; Zymed, South San Fransisco, CA, USA), followed by AP–conjugated streptavidin (1:1000 dilution; Jackson Immunoresearch Lab., Westgrove, PA), and finally ISC were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) insoluble alkaline-phosphatase (AP) substrate (Sigma–Aldrich). The frequency of CpG-specific ISC per 1×10^6 cells was calculated by subtracting the number of spots present in wells stimulated with GpC controls and media from the number of spots present in CpG-ODN stimulated wells. An inverted light microscope was used to count three replicate wells for each cell population and data presented are mean values for replicate samples. To inhibit the action of IL-10, neutralizing mouse anti-bovine IL-10 antibody (Serotec, MCA 2111EL) was added at 1/200 dilution with CpG ODN to cell cultures.

5.3.7 Magnetic activated cell sorting (MACS)

The $CD21^+$ B cell fraction was isolated as described previously [246]. Briefly JPP and PBMC were stained with mouse anti-bovine antibody (IgG1, AbD Serotec, UK) for 15 min at 4^0C. The cells were then washed twice with MACS buffer (PBSA,

0.5M EDTA and 10% BSA) and followed by centrifugation for 8 min at 440 x g. The cells were then stained with goat anti-mouse IgG1 phycoerythrin (PE) conjugate (Southern Biotech, A1, USA) for 15 min at 4^0C and wash again as above. The cells were then labeled with anti-PE magnetic beads for 15 min at 4^0C and eluted through the LC MACS column (Miltenyi Biotec, Bergish Gladbach, Germany) according to manufacturer's instruction. The $CD21^+$ B cell fraction was eluted, washed in PBSA and re-suspended in AIM V media containing 2% FBS. The purity of the cells was above 94% as assessed by flow cytometry.

5.3.8 Quantitative RT-PCR

RT-PCR was used to quantify TLR9 mRNA in IPP, JPP, mLN, jejunum (small intestine between PP), duodenum, and colon. TLR9 mRNA was also assessed in single cell suspensions prepared from these tissues [IPP, JPP, mLN, and PBMC].

5.3.8.1 mRNA isolation and cDNA synthesis

Total RNA was isolated from either homogenized tissues (IPP, JPP, mLN) or from isolated cell suspensions (PBMC, IPP, JPP, mLN) using Trizol reagent (Invitrogen, Carlsbad, CA) and RNA samples were treated with DNAse I Amp Grade (Invitrogen) (1 U/μg of RNA). The absence of genomic DNA contamination was validated by use of treated RNA as template directly in PCR. No amplified product was present. RNAs were quantified by determining optical density at 260 nm (OD_{260}) and the OD_{260}/OD_{280} ratio was calculated to assess purity. Further, RNA quality was assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer). For complementary DNA (cDNA), RNA (1 μg) was incubated in a final volume of 15 μl with dNTP (0.5 mM final each) (Invitrogen), 0.5 μg oligo dT (Invitrogen), RNAse out inhibitor (20 U) (Invitrogen), Superscript ^{TM} reverse transcriptase (RT) (8 U) (Invitrogen), and 1× buffer RT (Invitrogen). The reaction was allowed to proceed for 40 mins at 42 °C and

then heat inactivated at 93 °C for 5 min. The cDNA generated was either used immediately for qPCR or stored at −80 °C.

5.3.8.2. Real-time RT-PCR

The cDNA generated as above were used in the Real-time PCR. cDNA was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. All primers were designed using Clone Manager (Scientific and Educational Software, Cary, NC). TLR9 forward primer 5' CGTGAGCAGCAACAGCATC 3' and reverse primer 5' TAAGCGACCGAACCAGAAGG 3' were purchased from Sigma–Aldrich. The PCR conditions were 95 °C for 3 min, followed by 45 cycles with denaturation at 95 °C for 15 s, annealing temperature at 59°C for 30 s, and elongation at 72 °C for 30 s. Real-time assays were run on a Bio-Rad iCycler iQ system (Bio-Rad, Hercules, CA). The specificity of the PCR reactions was assessed by the analysis of the melting curves of the products and size verification and sequencing of the amplicons. To normalize the amount of cDNA, we sampled equal tissue sizes, quantitated RNA, assessed its quality prior to reverse transcription, and used a reference gene. Samples were normalized internally using the average cycle threshold (Ct) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference in each tissue. To quantify the numbers of copies, plasmid containing sheep TLR9 cDNA were linearized with *Xho*I (New England Biolabs) and 10 two-fold dilutions of the plasmid were used to create a standard curve for quantification of the RNA-generated cDNA. Values were expressed as transcript copy number per 1µg of total input RNA, which were determined in each sample by interpolation with the respective standard curves. The correlation coefficient of TLR9 standard curve was 0.996. The concentration of the test samples were calculated from the standard curves, according to the formula $y = -MC_t + B$, where M is the slope of the curve, C_t the point during the exponential phase of amplification in which the fluorescent signal is first recorded as being statistically significant above background, and B is the y-axis intercept. Only C_t values <40 were used for calculation of the PCR

efficiency from the given slope according to the equation: PCR efficiency = $(10^{[-1/M]} - 1) \times 100$. All PCRs displayed efficiency between 94% and 100%.

5.3.9 Statistical analysis

Data were analyzed using the statistical software program GraphPad Prism 5 (Graphpad, San Diego, CA, USA). One way ANOVA (Kruskal-Wallis) and Dunn's multiple comparison tests were performed on each treatment and tissue. Differences were considered significant when $p<0.05$.

5.4. Results

5.4.1 IFNα and IFNγ secretion is reduced in Peyer's patch cells stimulated with CpG ODN

We determined IFN responses of IPP and JPP cells stimulated with 2 μg/mL of CpG ODN and compared responses to those of LN cells. Both scLN and mLN exhibited IFNα responses that were significantly greater ($p<0.05$) than both JPP and IPP cells (Fig. 5.1 A). The IFNα responses of JPP cells were not significantly different from IPP. Stimulation of scLN and mLN cells with CpG ODN resulted in a significantly higher production (5-10 fold) of IFNγ than cells from either ileal of jejunal PP (Fig. 5.1 B). However, JPP cells produced significantly more IFNγ when compared to IPP cells (Fig. 5.1 B). Thus CpG-induced IFNα and IFNγ secretion by Peyer's patches cells was significantly reduced when compared to LN cells.

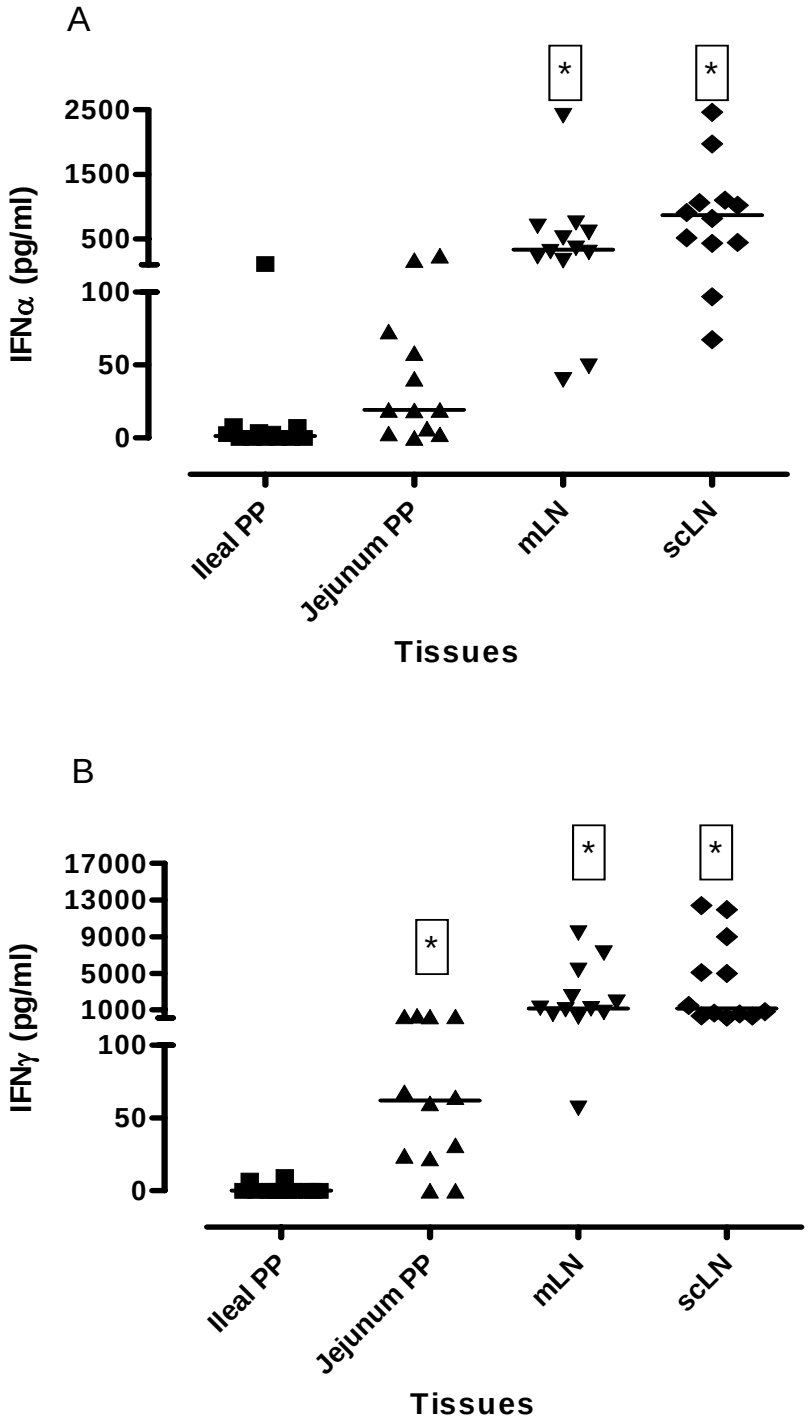

Fig. 5.1 A-B: IFNα and IFNγ secretion by ovine IPP, JPP and LN cells following stimulation with 2.0 μg/mL of CpG ODN (2429). Data represent individual values for 12 animals (n=12). Horizontal bar represents mean value for each tissue and significant difference ($p<0.05$) relative to ileal Peyer's patches are represented by boxed *.

5.4.2 Reduced cytokine responses of PP cells are not due to suboptimal CpG ODN concentration.

In the experiment described above, the concentration of CpG ODN used was 2 μg/mL as this was reported to be optimal for induction of immune responses in LN and PBMC [203, 238]. We reasoned that PP cells may require a higher dose of CpG ODN for optimal response. For this reason, we performed a dose titration experiment using CpG ODN concentrations of 0 .66, 2.0, 6.0 and 18.0 μg/mL.

As shown in Fig. 5.2 A, IPP cells did not secrete detectable IFNα when stimulated with increasing concentrations of any of the three classes of CpG ODN. Although with JPP cells both 2 and 6 μg/mL of CpG ODN induced production of IFNα, there was no significant difference between the two ODN concentrations (Fig. 5.2 B).

All three classes of CpG ODN induced IFNα secretion by mLN cells (Fig. 5.2 C). The optimal dose of CpG ODN was 2 μg/mL and increasing the concentration of ODN did not significantly enhance the response. Similarly, with PBMC, 2 μg/mL of CpG ODN induced an optimal IFNα response and increasing concentration of ODN did not significantly increase the response (Fig. 5.2 D).

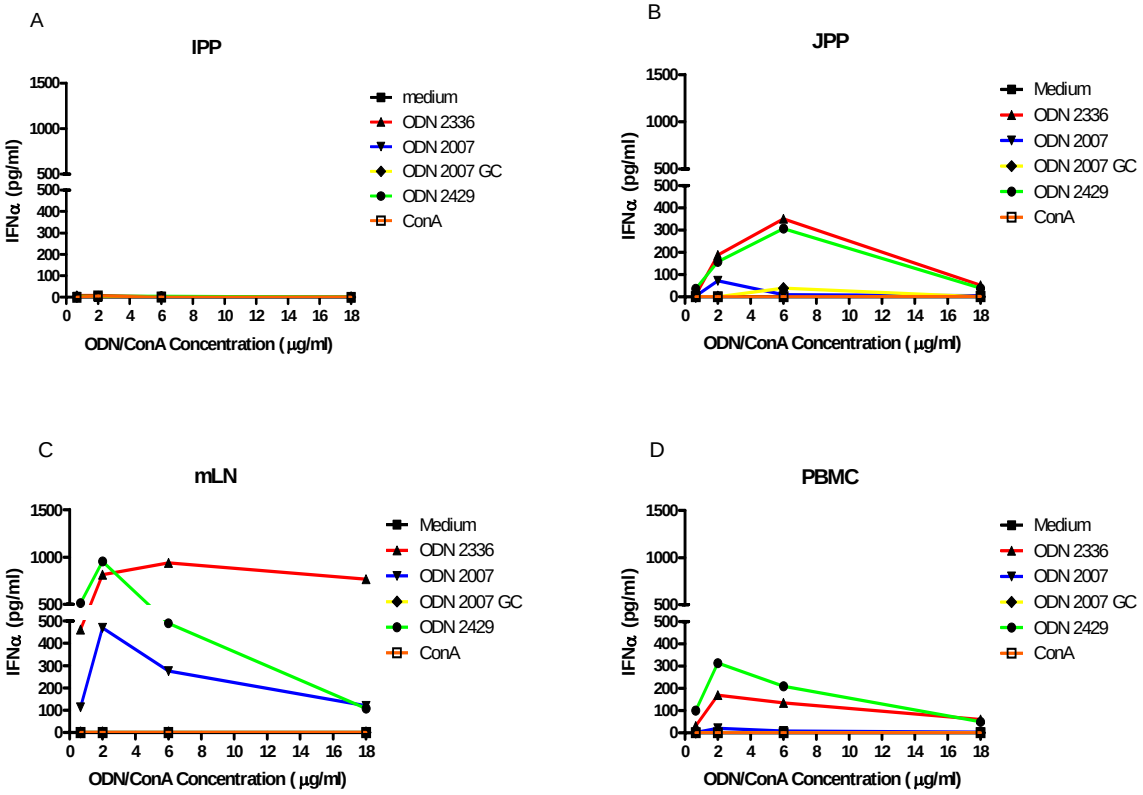

Fig. 5.2 A-D: IFNα secretion by ovine IPP, JPP, mLN cells and PBMC following stimulation with varying concentrations of all three classes of CpG ODN (A: 2336, B: 2007 and C: 2429). Data represent median values for cells isolated from 8 animals and assayed with each concentration of CpG ODN.

IFNγ secretion in response to various concentrations of CpG ODN followed a pattern similar to that seen for IFNα. IPP cells did not produce detectable levels of IFNγ regardless of the type and concentration of CpG ODN used for stimulation (Fig. 5.3 A). Concavalin A (Con A) stimulation confirmed that the cells were viable and capable of secreting IFNγ (Fig. 5.3 A-D). Con A induced IFNγ but not IFNα (Fig. 5.3 A-D) from all tissues tested. JPP cells did secrete detectable levels of IFNγ when stimulated with 2, 6 and 18 µg/mL of CpG ODN but the responses were not significantly different with each concentration (Fig. 5.3 B). A- and C-class CpG ODN induced JPP to secrete higher levels of IFNγ higher than B-class (Fig. 5.3 B) and all three classes of CpG ODN

induced similar IFNγ responses in mLN cells (Fig. 5.3 C). The optimal CpG ODN dose was between 2-6 µg/mL of ODN for mLN cells.

PBMC showed low IFNγ responses when stimulated by A- and C-class of CpG ODN (Fig. 5.3 D) as previously reported [238]. Therefore regardless of the concentration or class of CpG ODN, interferon secretion (IFNα and IFNγ) by PP cells was significantly lower when compared to cells isolated from LN.

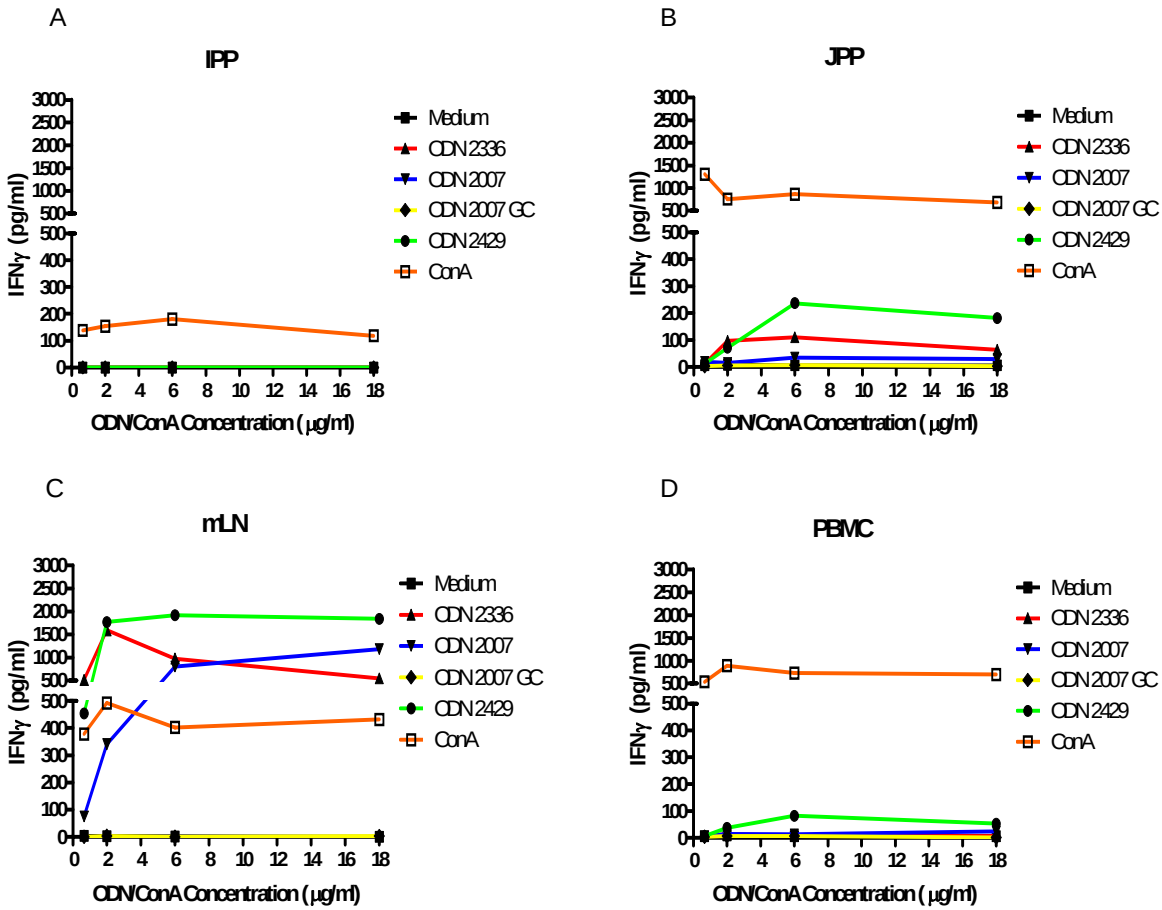

Fig. 5.3 A-D: IFNγ secretion responses by ovine IPP, JPP, mLN cells and PBMC following stimulation with varying concentrations of all three classes of CpG ODN (A: 2336, B: 2007 and C: 2429). Data represent the median value for cells isolated from 8 animals and assayed at each concentration of CpG ODN.

5.4.3 IL-12 secretion is also reduced in PP stimulated with CpG ODN

It has been recently reported that pDC in mouse Peyer's patches stimulated with CpG ODN do not produce IFNα but secrete significant amount of IL-12 [143]. Therefore we investigated whether CpG ODN could induce detectable IL-12 secretion by sheep PP cells.

As shown in Fig. 5.4 A, IPP cells did not secrete significant levels of IL-12 when stimulated with B- and C-class CpG ODN, but these ODNs induced significant IL-12 production by JPP cells (Fig. 5.4 B). This response was CpG-specific as the GpC control did not induce any IL-12 (Fig. 5.4 B).

When comparing IL-12 secretion among tissues stimulated by B- and C-class CpG ODN (Fig. 5.4 C-D), secretion of IL-12 was significantly lower with IPP cells than the other three tissues. Following stimulation by CpG ODN, mLN secreted significantly higher levels of IL-12 than PBMC and JPP (Fig. 5.4 C-D). In turn, PBMC secreted higher levels of IL-12 than JPP when stimulated with CpG ODN (Fig. 5.4 C-D). Therefore, IL-12 secretion by PP cells was lower than cells isolated from other tissues and followed a trend similar to the IFNα and IFNγ responses.

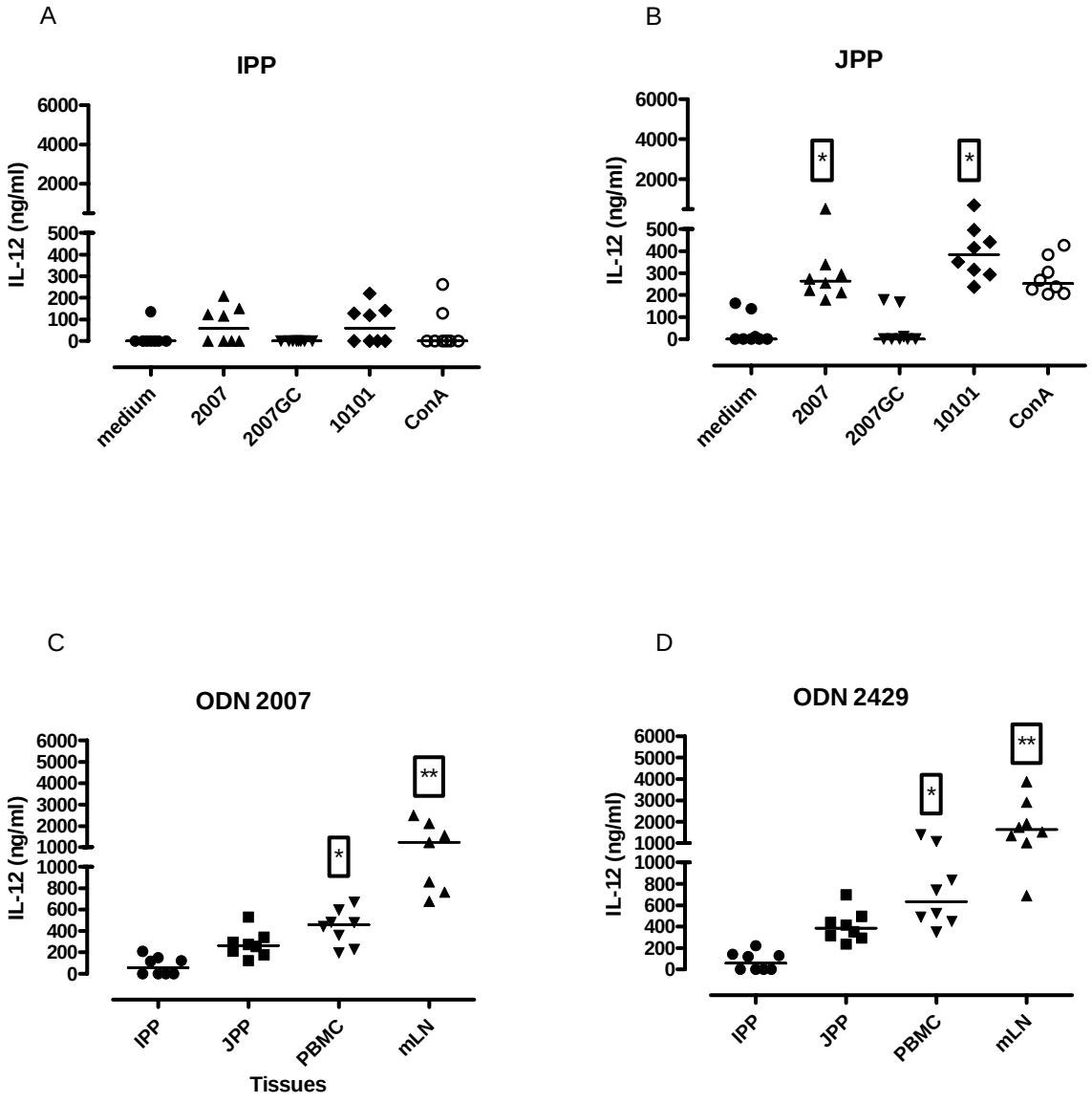

Fig. 5.4 A-D: IL-12 secretion by ovine IPP, JPP, mLN cells and PBMC following stimulation with B- and C-class CpG ODN (B: 2007 and C: 2429) at 2 µg/mL. Data represent the mean value for cells isolated from 8 animals. Significant difference ($p<0.05$) relative to (i) GpC ODN in Fig. 5.4 A-B and (ii) ileal Peyer's patches in Fig. 5.4 C-D are represented by boxed *. ** Significant difference relative to both PP.

5.4.4 Peyer's patches have a lower frequency of IFNα producing cells

We hypothesized that the reduced IFNα response in PP is a consequence of either a lower frequency or a lack of IFNα-secreting cells in the tissue. Therefore we assessed the number of IFNα producing cells in the various tissues using an ELISPOT assay.

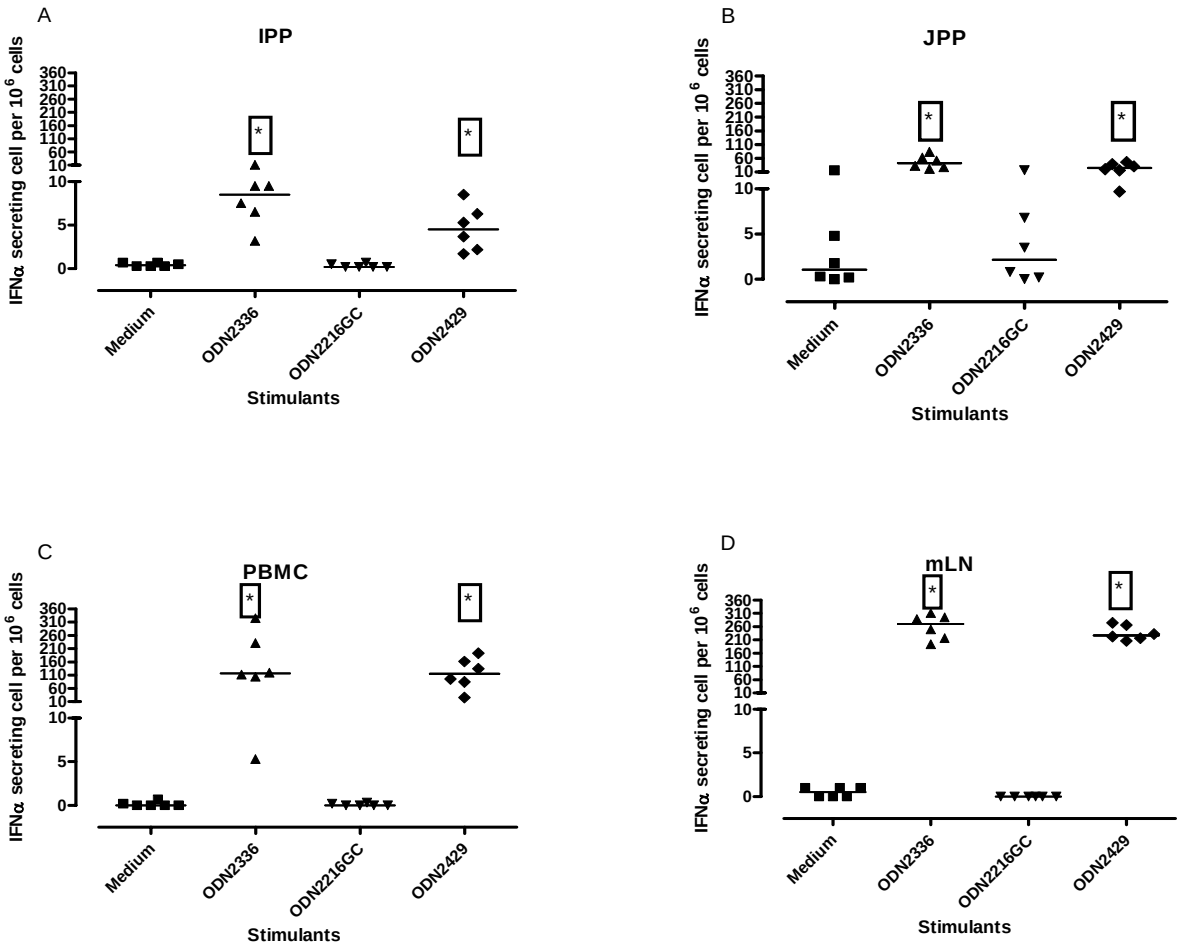

Fig. 5.5 A-D: Frequency of IFNα producing cell in ovine IPP, JPP, PBMC, and mLN following stimulation with either 2 µg/mL of CpG ODN (A: 2336; C: 2429), 2216GpC or medium alone. Values for cells from individual animals are presented with mean value indicated by horizontal bar for each treatment group (n=6). Significant difference in IFNα producing cells per million cells relative to GpC controls are indicated by boxed * ($P<0.05$).

A significant number of IFNα–secreting cells were detected in IPP following stimulation with CpG ODN versus medium alone (Fig. 5.5 A). This response was CpG specific as the GpC control did not induce any detectable responses (Fig. 5.5 A). Similarly, significant numbers of IFNα-secreting cells were detected in JPP, PBMC and mLN stimulated with CpG ODN (Fig. 5.5 B-D).

When comparing among tissues, following CpG ODN stimulation (Fig. 5.6), it was found that the frequency of IFNα-secreting cells was significantly lower in IPP than all the other tissues. mLN contained significantly higher IFNα–secreting cells than PBMC and JPP. In turn, PBMC had a significantly higher frequency of IFNα-secreting cells than JPP following stimulation with CpG ODN (Fig. 5.6). ELISPOT analysis of ISC frequency was consistent with data for ELISA analysis of cytokines (Fig. 5.1 A).

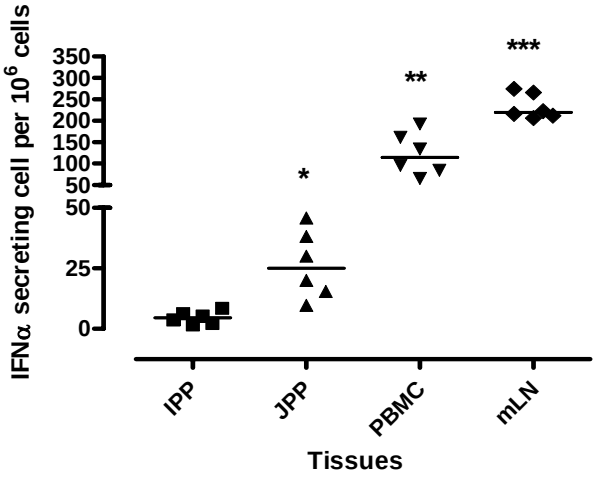

Fig. 5.6: Frequency of IFNα producing cells in ovine IPP, JPP, PBMC, and mLN following stimulation with 2 µg/mL of CpG ODN (2429). Data for individual animals are presented with mean value indicated by horizontal bar for each group (n=6). Significant differences in IFNα producing cells relative to IPP are indicated by * ($P<0.05$). ** represent significant difference between PBMC and both PP whereas *** represent significant difference between mLN and all other tissues.

5.4.5 Peyer's Patches express high levels of TLR9 mRNA.

All the data so far (Fig. 5.1-5.6) clearly indicated that IPP and JPP responses to CpG ODN stimulation were significantly lower when compared to cells isolated from LN regardless of CpG ODN concentration or class. We reasoned that IPP and JPP cells

may lack sufficient TLR9 expression to respond effectively to CpG ODN stimulation. Therefore, we evaluated TLR9 mRNA expression in cells isolated from different tissues. RNA isolated from IPP, JPP, blood and mLN tissue all expressed significantly higher copy numbers ($p<0.05$) of TLR9 than duodenum, jejunum and colon (Fig. 5.7 A). Interestingly, IPP and JPP tissue expressed similar levels of TLR9 mRNA as mLN and PBMC.

Since the CpG stimulation experiments were done with cells suspensions isolated from tissues, we wondered whether TLR9 expression in those cell suspensions differed from that in intact tissues.

As shown in Fig. 5.7 B, cell suspensions had significantly higher expression of TLR9 mRNA than intact tissues but the overall pattern of expression was similar. Thus these data confirmed that the cell populations used expressed TLR9. Therefore, poor CpG-induced interferon responses in PP were unlikely due to lack of TLR9 receptor expression (Fig. 5.7 A-B).

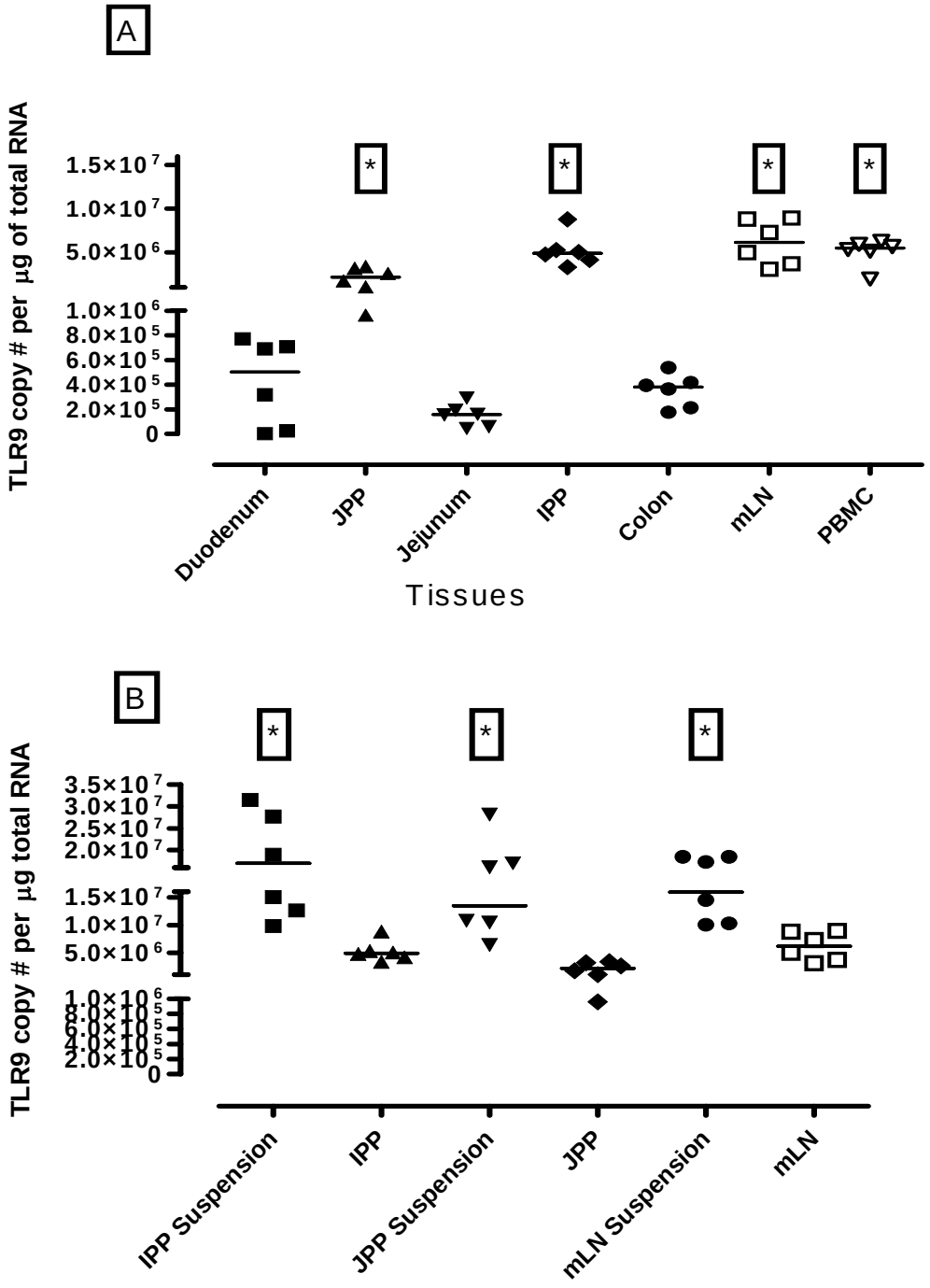

Fig. 5.7 A-B: TLR9 mRNA expression in ovine IPP, JPP, PBMC, mLN, duodenum, jejunum and colon. Data for individual animals are presented with mean value indicated by horizontal bar for each treatment group (n=6). Significant TLR9 mRNA expression relative to duodenum and colon is indicated by a boxed * ($P<0.05$). **B:** TLR9 mRNA expression in ovine IPP, JPP, mLN and their respective cells suspension. Data for individual animals are presented with mean value indicated by horizontal bar for each treatment group (n=6). Significant TLR9 mRNA expression in the cells suspension relative to their respective tissue is indicated by a boxed * ($P<0.05$).

5.4.6 Role of IL-10 in regulation of CpG-induced responses in Peyer's Patches

5.4.6.1 Kinetics of IL-10 production in PP

One factor which could contribute to the reduced CpG ODN induced cytokine responses by PP cells could be the presence of immunoregulatory cytokines such as IL-10. Therefore we evaluated the kinetics of IL-10 secretion in cultures of IPP, JPP, mLN cells and PBMC at different time points (6, 16, 24 and 48 hrs) following stimulation with CpG ODN. In PBMC and mLN cultures, significant IL-10 was not detected until after 48 hrs (Fig. 5.8 C-D) whereas with JPP and IPP cells, significant IL-10 was detected as early as 16 hrs of culture (Fig. 5.8 A-B) when stimulated with CpG ODN. In fact, IPP cells had detectable levels of IL-10 as early as 6 hrs of culture (Fig. 5.8 A) and the level of IL-10 production was higher than all other tissues at all time points (Fig. 5.8 A-D).

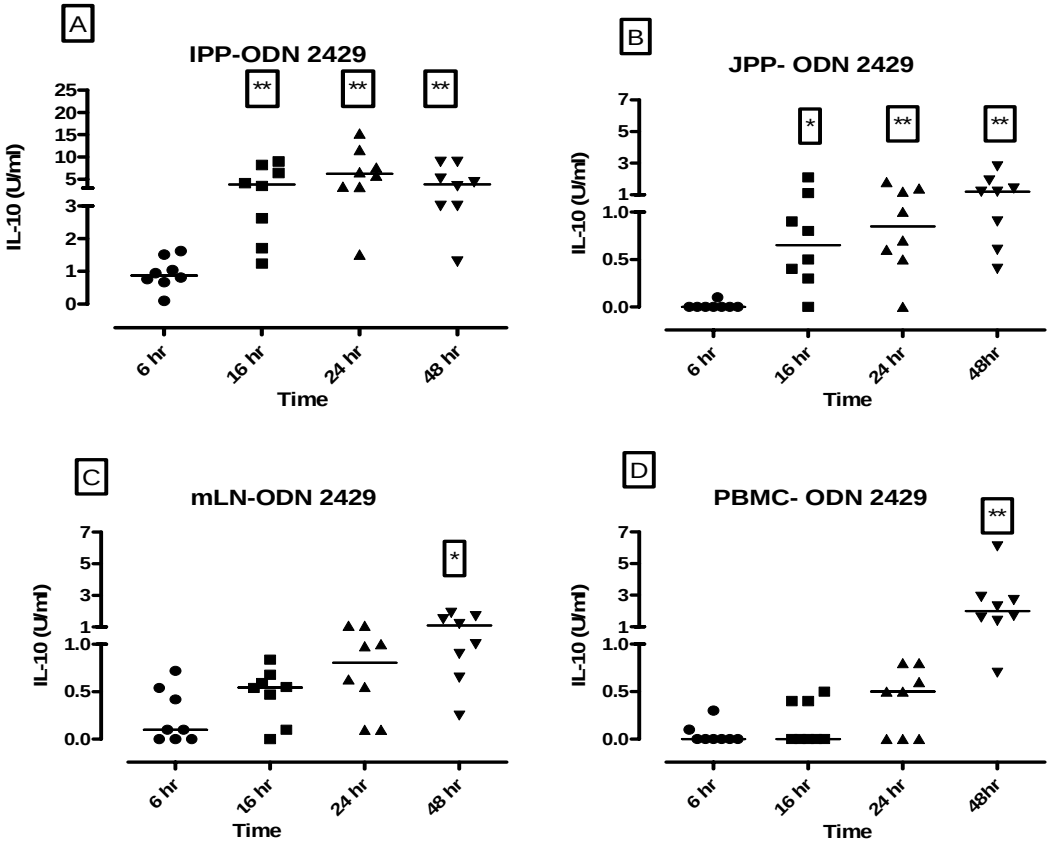

Fig. 5.8 A-D: Kinetics of IL-10 secretion by IPP, JPP, mLN and PBMC stimulated with CpG ODN (2429). Data for individual animals are presented with mean value indicated by horizontal bar for each treatment group (n=8). Significant difference in IL-10 secretion relative to 6 hrs time point is indicated by * $p<0.1$ and ** $p<0.05$. U/mL for IL-10 was previously defined by Kwong et al [249], as the biological activity of IL-10 which is represented by one unit being the reciprocal of the dilution that inhibited IFNγ by 50%.

Surprisingly, we observed that unstimulated PP cells produced detectable levels of IL-10 whereas with PBMC detectable IL-10 production occurred only after CpG ODN stimulation (Fig. 5.9). Therefore, PP cells spontaneously secreted IL-10 as early as 6 hrs in cultured cells.

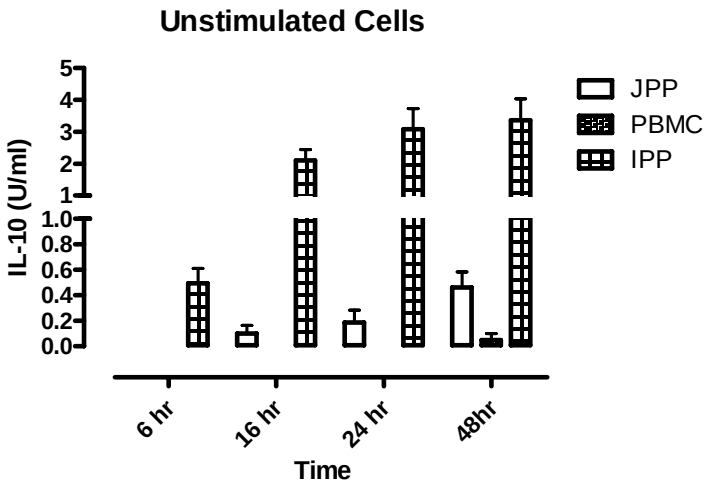

Fig. 5.9: Kinetics of IL-10 secretion by IPP, JPP, mLN and PBMC cultured in medium.

5.4.6.2 IL-10 inhibits IFNα production in PP

The kinetics of IL-10 production indicated that this cytokine was secreted very early in PP cell cultures. Therefore, we hypothesized that IL-10 may suppress the induction of IFNα. We evaluated the effect of neutralizing IL-10 on CpG-induced IFNα response. The optimal concentration of neutralizing IL-10 antibody was evaluated by a titration experiment and 1/200 dilution of the antibody was found to be optimal (Data not shown). As previously observed, CpG ODN (2429) alone induced IFNα-producing cells (IPC) in IPP, JPP and PBMC (Fig. 5.10 A-C). Neutralization of IL-10 resulted in a significant increase in CpG-induced INFα producing cells in IPP, JPP (Fig. 5.10 A-B) but not PBMC (Fig. 5.10 C). Therefore IL-10 appeared to play a direct role in inhibiting IFNα secretion by PP cells.

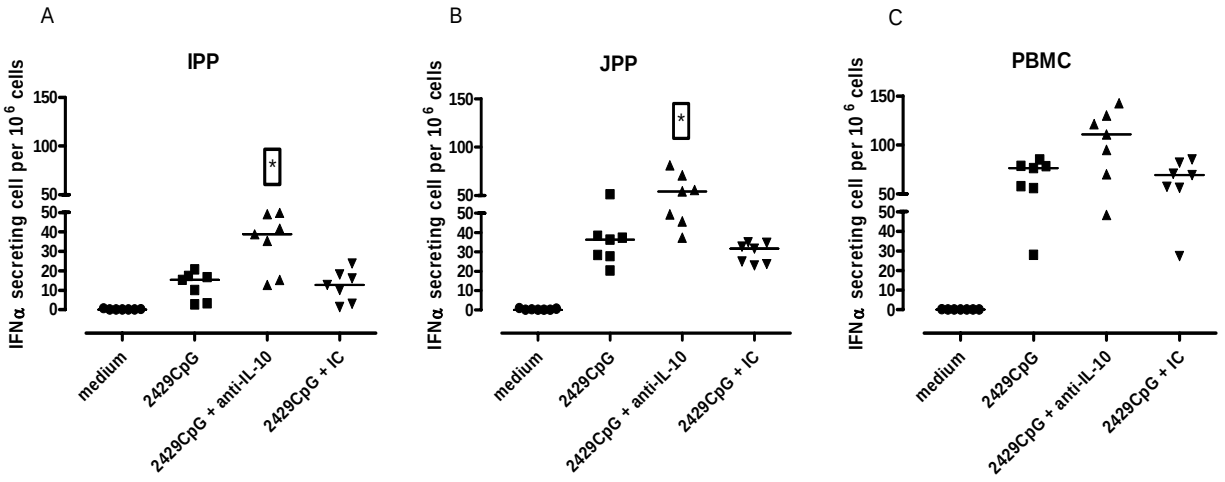

Fig. 5.10 A-C: IFNα producing cells responses in ovine IPP, JPP and PBMC following stimulation with 2 µg/mL of CpG ODN (C: 2429) and/or anti-IL-10 antibody (1/200) or isotype control antibody (IC) or media alone. Data for individual animals are presented with mean value indicated by horizontal bar for each treatment group (n=7). Significant differences in IFNα producing cells per million cells relative to 2429CpG + IC are indicated by boxed * ($P<0.05$).

5.4.7 Peyer's patch CD21$^+$ B cells are the major source of the IL-10

We then investigated what was the predominant cell type in PP responsible for IL-10 production. As shown before, JPP cells secreted IL-10 regardless of CpG ODN stimulation (Fig. 5.11 A) whereas PBMC secreted IL-10 only after CpG stimulation (Fig. 5.11 D). When MACs purified CD21$^+$ B cells from both tissues were stimulated with CpG ODN, we observed that unstimulated JPP CD21$^+$ B cells secreted IL-10 (Fig. 5.11 B) whereas PBMC CD21$^+$ B cells did not secrete IL-10, even after CpG ODN stimulation (Fig. 5.11 E). Induction of IL-10 in PBMC may require involvement of more than one cell type since neither of the fractionated PBMC populations produced IL-10 (Fig. 5.11 D-F).

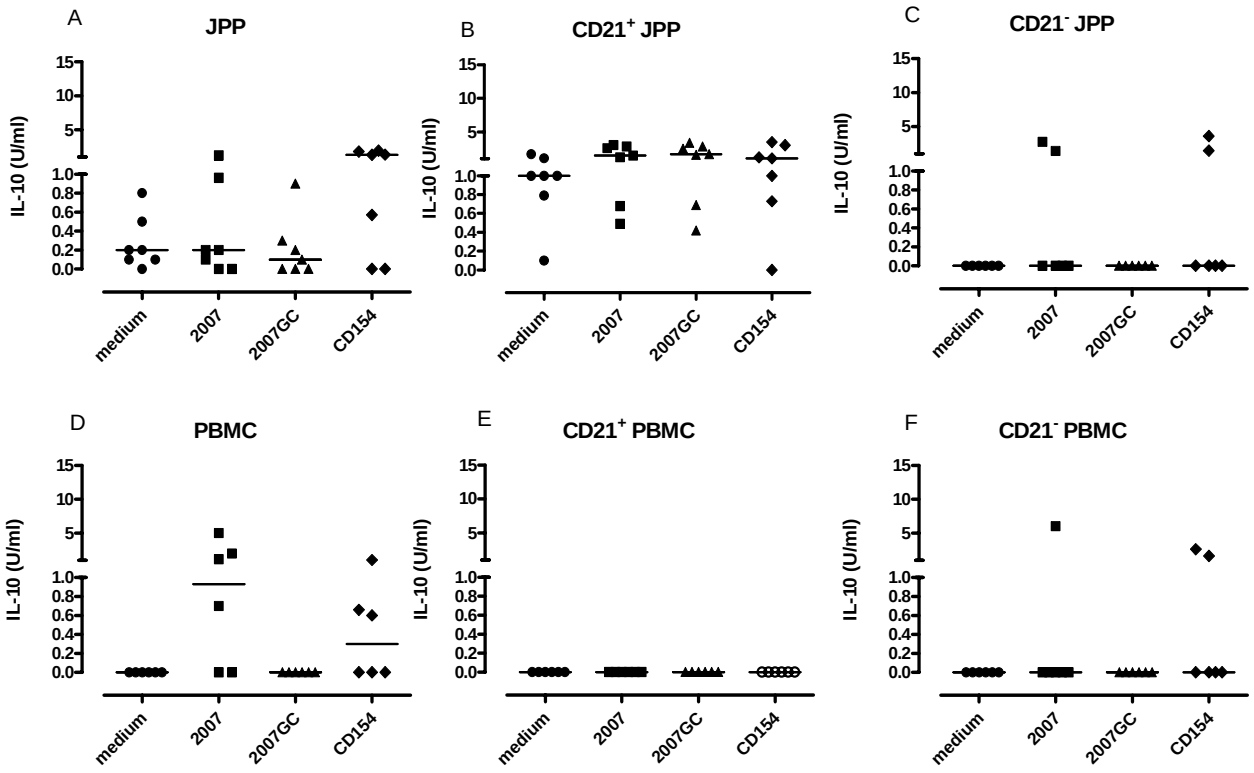

Fig. 5.11 A-F: Using MACs isolation, purified CD21$^+$ B cells from JPP and PBMC were obtained. JPP CD21$^+$ B cells secrete IL-10 spontaneously whereas PBMC CD21$^+$ B cells do not. Data represent the mean of 7 animals for IL-10.

5.4.8 CD21$^+$ Peyer's patch B cells inhibit CpG-induced IFNα, IFNγ and IL-12 responses in JPP.

We depleted CD21$^+$ PP B cells from JPP cells and using equivalent cell numbers, we tested whether CD21$^+$ cells had any effect on the CpG-induced responses in JPP. As shown in Fig. 5.12 A, IFNα responses in JPP CD21$^-$ cells were significantly higher than in JPP upon stimulation with CpG ODN 2429 whereas no IFNα was secreted by CD21$^+$ cells. Similar results were obtained when CpG-induced IFNγ and IL-12 responses were assessed in CD21$^-$ cells compared to JPP (Fig. 5.12 B and 5.12 C). CD21$^-$ cells secreted significantly higher level of IFNγ and IL-12. These results suggest strongly that CD21$^+$ B cells play a regulatory role in this tissue.

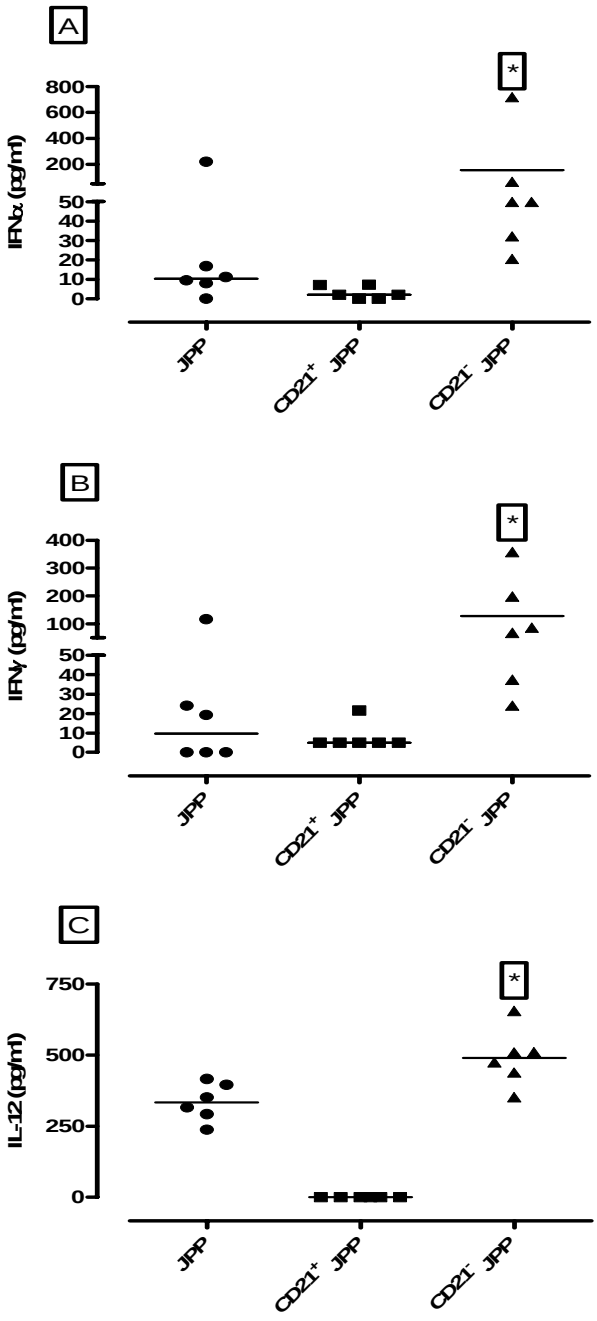

Fig. 5.12 A-C: Using MACs isolation, $CD21^+$ B cells were depleted from JPP. JPP, JPP-$CD21^+$ B cell, JPP-$CD21^-$ cell fractions were assessed for IFNα, IFNγ and IL-12 following 5μg/ml of CpG ODN (2429). Data represent the mean of 6 animals. Boxed * represent significant difference ($p<0.05$) between $CD21^-$ fraction and whole JPP cells.

5.4.9 Characterization of CD21$^+$ B cells from JPP and PBMC by FACs analysis

Phenotypic characterization of JPP B cells by flow cytometry revealed that the IL-10-secreting B cells were CD21$^+$ CD72$^+$MHCII$^+$ (Fig. 5.13).

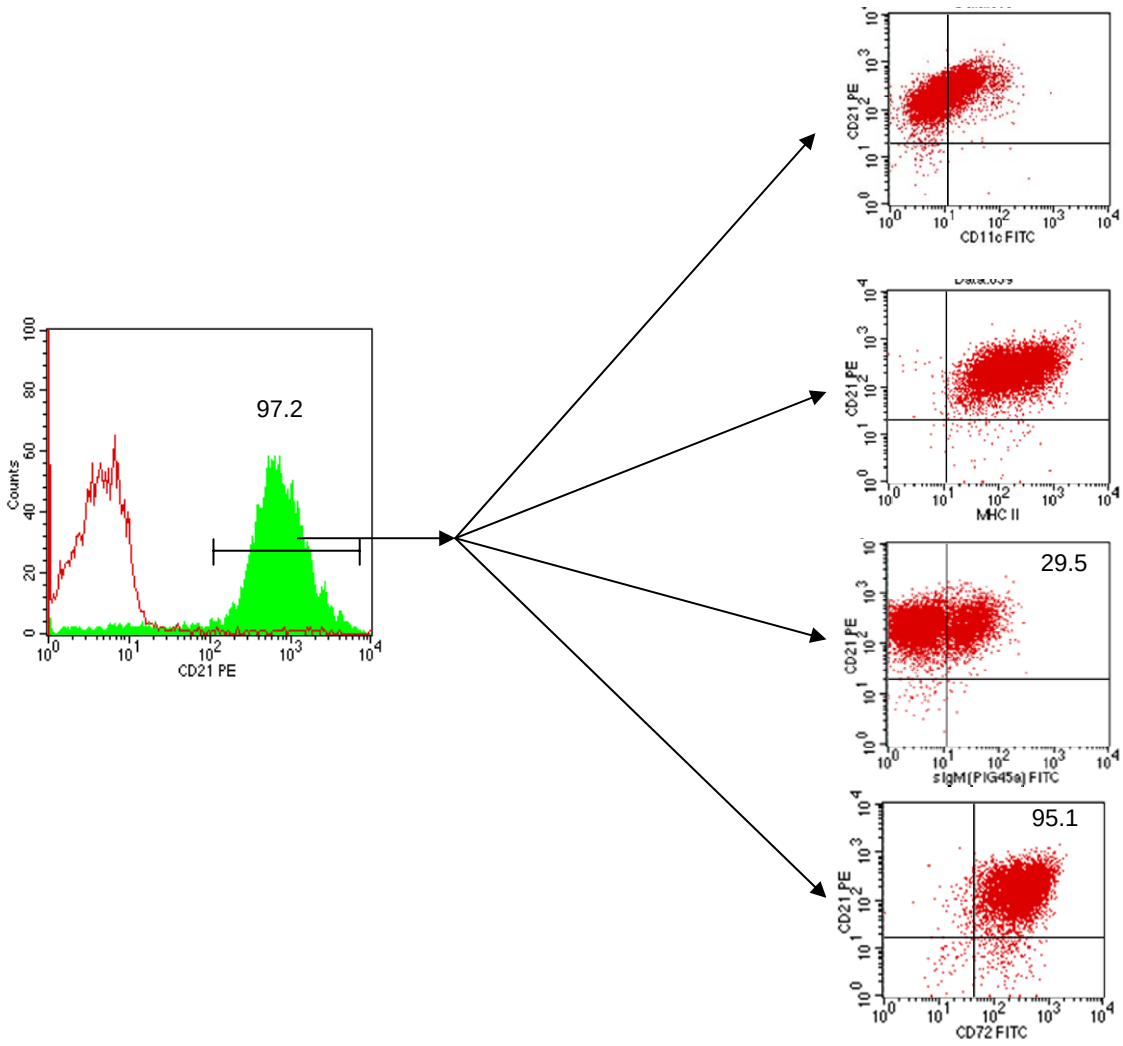

Fig. 5.13: Isolated JPP CD21$^+$ B cells with purity of 97.2% co-labelled with anti-CD11c, MHCII, sIgM and CD72 FITC.

Further phenotypic characterization of PBMC $CD21^+$ B cells by flow cytometry revealed that the B cells were $CD21^+$ $CD72^+CD11c^+MHCII^+sIgM^+$ (Fig. 5.14).

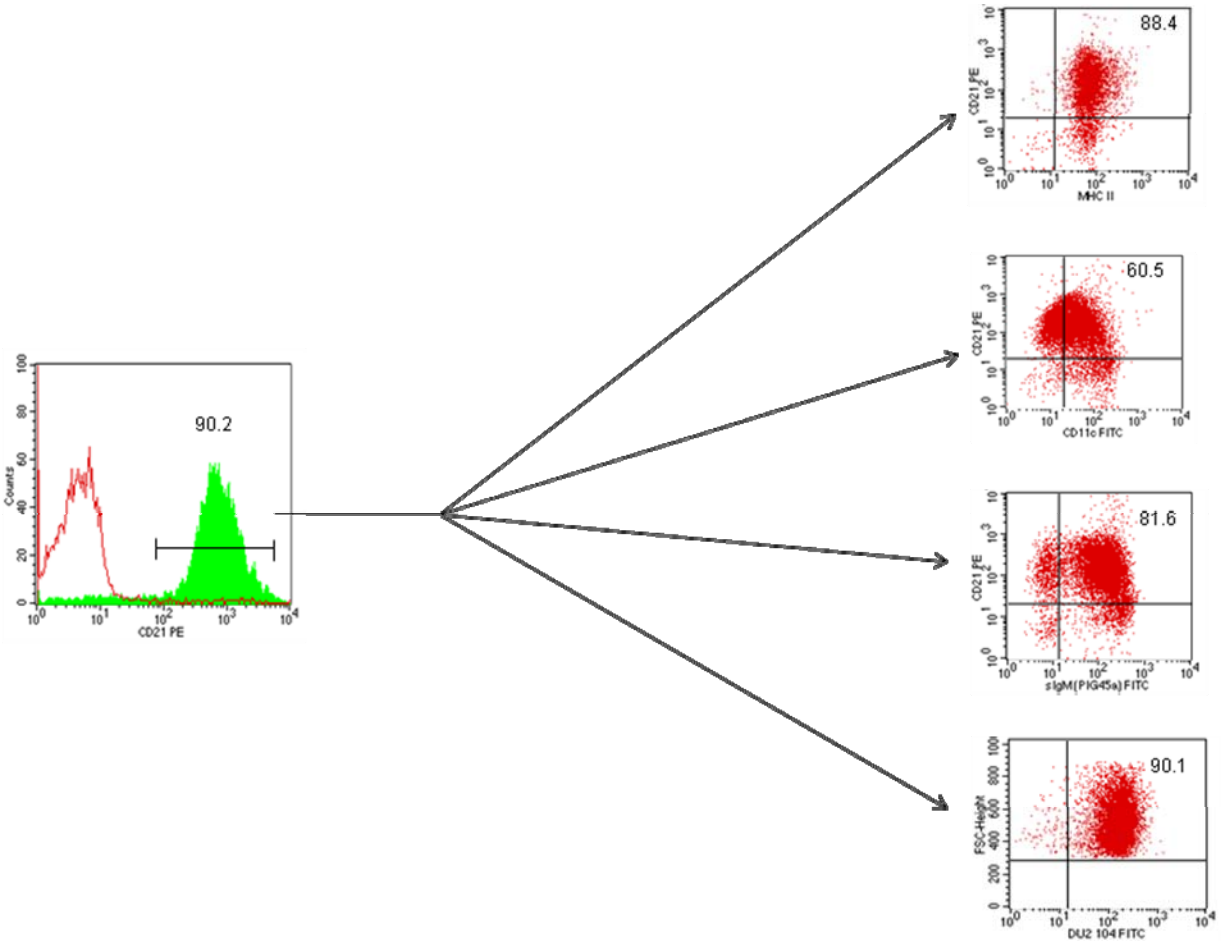

Fig. 5.14: Isolated PBMC $CD21^+$ B cells with purity of 90.2% co-labelled with anti-CD11c, MHCII, sIgM and CD72 FITC.

5.5 Discussion

Our investigations revealed that JPP $CD21^+$ B cells secrete IL-10 which plays a direct role in the suppression on IFNα secretion by JPP cells. Moreover, this B cell population spontaneously secretes IL-10 in the absence of TLR stimulation.

In sheep, $CD21^+$ B cells are representative of naïve B cell population and are mainly $CD5^-$, $CD11b^-$, $CD11c^-$, $sIgM^{lo}$ and $CD62L^+$ [269]. They localize in the B cell follicles of Peyer's patches, lymph nodes and spleen and are actively recirculating in blood and lymphatic system [112, 270, 271]. In this study, we found that unstimulated $CD21^+$ B cells from PP cells secrete high amount of IL-10 whereas unstimulated $CD21^+$ B cells from blood do not secrete IL-10. The IL-10-secreting $CD21^+$ B cells in Peyer's patches may represent a novel population of B cells analogous to the recently proposed regulatory B cells (B_{regs}) [140, 152, 154]. Typically B cells are known mainly for producing antibodies but they have the ability to produce cytokines and function as antigen presenting cells. It is now known that like T cells, the B cell population consists of distinct subsets capable of performing pathogenic and regulatory functions [152]. B_{regs} have been observed in several murine models of chronic inflammation including inflammatory bowel disease, but not in normal intestinal tissue [152]. One of the mechanism by which B_{regs} mediate their regulatory functions is via production of the regulatory cytokines IL-10 and TGFβ which dampen inflammation [272-275]. Here, we propose a novel B cell population capable of secreting IL-10 in the steady state and which may perform regulatory functions similar to murine B_{regs}. However, there appears to be a major difference between the two: sheep PP B_{regs} constitutively occur in normal intestinal tissues in the absence of any known pathology and spontaneously secrete IL-10 in the absence of TLR9 stimulation. In contrast, murine B_{regs} are associated with chronically inflamed intestinal tissue and are not present in steady state [272]. Further investigation is required to characterize the function of these sheep PP B_{regs}.

Our investigation revealed that IL-10 directly suppressed IFNα responses in PP. IL-10 is an immunoregulatory and anti-inflammatory cytokine. The major known sources of IL-10 are dendritic cells, regulatory T cells and macrophages [116, 276, 277]. IL-10 can affect a variety of cell types including monocytes, macrophages, dendritic,

neutrophils. It potently inhibits production of a variety of cytokines (IL-1, IL-6, IL-12, IL-18, TNF) [278-281] and chemokines (IL-8, IP-10, Mip3α, Rantes) [282-284], thereby affecting recruitment of neutrophils, DC, and monocytes. IL-10 induces the generation of regulatory T cells that can suppress antigen specific responses in vivo and in vitro [285-287]. IL-10 also plays an important role in regulating mucosal responses as indicated by numerous studies with IL-10 deficient ($IL\text{-}10^{-/-}$) mice which develop spontaneous intestinal inflammation [139, 288, 289]. Our observation that IL-10 inhibited IFNα responses is consistent with Contractor *et al* (2007) who showed that IL-10 inhibited IFNα production spleen pDCs, and also implicated IL-10 in the inability of pDCs from PP to produce IFNα although they did not evaluate the direct effect of IL-10 on PP pDCs. Recently Monteleone et al., 2008 showed that lamina propria DC failed to secrete IL-12 in response to CpG and this was attributed to inhibitory IL-10 secreted by these cells and the authors also showed that unlike spleen or LN, mucosal DC expressed high levels of TLR2, 3, 4, 5 and 9 but responded poorly to agonists for these TLR [267]. However this study did not investigate the effect of IL-10 on IFNα in DC or other cells in PP.

IFNα, a type I interferon, is known for its role in antiviral defences, but can also activate innate and adaptive immune responses [290]. IFNα, together with IL-12 can induce NK cell cytotoxicity and also augment Th1-mediated immunity through innate production of IFNγ by NK cells and $CD8^+$ cells. IFNα can also act independently of IL-12 to activate cDC maturation and cross-presentation and to enhance $CD8^+$ T cell responses. Therefore, inhibition of IFNα in the PP may skew the balance of immune responses from potentially damaging Th1 type responses.

JPP but not IPP are the primary sites where immune responses are initiated in the gut [110]. We would therefore expect that cells from PP would respond to a potent activator like CpG ODN in a manner similar to other secondary lymphoid tissues like LN. In this study we show that CpG-induced responses (IFNα, IFNγ, and IL-12) in PP are reduced compared to the LN. Our observations are in line with others who have reported that dendritic cells from lamina propria do not respond to TLR ligands unlike spleen DC [143, 168]. These results are not surprising as the intestine is constantly exposed to bacterial DNA and other MAMPs present in microbial flora and must have

mechanisms for preventing unnecessary responses to microbial stimuli. Our findings that PP respond poorly to CpG ODN in terms of IFNα and IFNγ despite expressing high levels of TLR9 is consistent with observations by Pedersen and colleagues who reported that primary epithelial cells isolated from normal human colon expressed TLR9 mRNA, but were completely unresponsive to CpG-ODN stimulation *in vitro* [291]. One of the mechanisms that have been proposed for this tolerance is the downregulation of TLR receptors on mucosal immune cells, for example TLR4 in lamina propria macrophages [126]. However, we observed that TLR9 mRNA expression in both the IPP and JPP were abundant and comparable to expression in immune cells from LN and blood. The similar TLR9 expression profiles in both IPP and JPP and mLN in our studies are consistent with the TLR profiles reported recently in sheep [251] and in pigs [252]. Taken together, data from our studies and those from others suggest that the low responses of PP cells to CpG ODN were not due to downregulation of TLR9 receptor. Rather, other mechanisms are involved in downregulating CpG-induced responses.

In sheep and many other species including humans, two distinct PP are found in the small intestine namely JPP and IPP, each with their own characteristic development, structure and function described by Landsverk *et al* [235]. IPP consist of a continuous patch in ovine species which involutes at a young age and is thought to be responsible for the primary generation of B cells, similar to the bursa of Fabricus in avian species. In contrast, JPP persist in the adult animal and contain M cells with clusters of B cells in the follicle-associated epithelium [100, 235, 292]. The cell compositions of the PP are quite different from LN and PBMC. Unlike peripheral lymph nodes, B cells comprise a large proportion of lymphoid cells in the PP (up to 80% in JPP and even higher in IPP) of sheep [100, 114] whereas the B cells frequency in PBMC or LN is around 30-40% [238]. Although the responses in PP were lower than other lymphoid compartments, we showed that JPP is the primary site for initiation of immune responses (IFNα, IFNγ and IL-12) in response to CpG ODN whereas IPP does not secrete these cytokines in respond to CpG ODN. This correlates with previous findings that JPP but not IPP are inductive sites for immune responses in the intestine [110]. Therefore implying that IPP is more of a primary lymphoid tissue in sheep. Moreover, we observed significantly higher levels of IL-10 secretion earlier in IPP cell cultures than JPP cell cultures. This

may be due to the higher frequency of B cells in IPP than JPP. The role of IL-10 in IPP is unknown.

We demonstrated that the frequency of IFNα producing cells is lower in PP compared to LN and PBMC. The major source of IFNα upon stimulation with CpG ODN is plasmacytoid dendritic cell (pDC) in humans [92] and in sheep ([293]. The reasons why PP contain fewer IFNα producing cells (presumably pDC) that retain their capacity to respond to MAMPs remains unknown.

Interestingly, our results also showed that IL-12 was produced in similar fashion as IFNα in the tested immune compartments (IPP, JPP, mLN and PBMC) when stimulated with CpG ODN. This observation suggests that IL-12 secretion is also dependent on the frequency of responder cells (probably pDC) in the tissues tested. Thus, the CpG-responder cells (presumably "pDC") in the Peyer's patches have the ability to produce both significant levels of IFNα and IL-12 similar to previous studies [67, 294] but different to a recent study [143].

One current explanation for immune homeostasis in the gut involves the ability of intestinal epithelial cells (IEC) to play a fundamental role involving different mechanisms (reviewed by [295]. For example, certain luminal bacterial products support colonic homeostasis via activation of epithelial surface TLRs in colonic epithelium [103, 134, 296]. The main distinction from our report is that these studies focus on the intestinal epithelial cell and TLR expression. Instead our report deals with immune cells that are present in PP. We clearly demonstrated that these PP immune cells were less responsive than their counterpart in other immune compartments. These results suggest that the immune cells in PP may have regulatory mechanisms to PAMPs independently of IEC. However, we are not excluding the effect of IEC as demonstrated by many studies.

While IL-10 downregulates IFNα responses, several other factors could also regulate PP cells responses to CpG ODN including; (i) additional anti-inflammatory cytokines such as transforming growth factor beta (TGFβ) and thymic stromal lymphopoietin (TSLP) contributing to the anti-inflammatory environment; (ii) Multiple signals may be required to induce optimal innate immune responses (e.g multiple TLR, BCR engagement, T cell and myeloid DC cooperation); (iii) regulatory T cell working

in concert with regulatory B cells in the intestine to modulate immune responses. However, further investigations are required to determine the mechanisms by which PP disregard PAMPs from commensal microorganisms but induce an immune response to pathogens.

5.6 Conclusion

The current analysis demonstrated that PP B cells spontaneously secrete IL-10 which "conditions" an anti-inflammatory environment in this tissue leading to poor cytokine responses to TLR9 agonist, CpG ODN. This may represent a strategy by which PP dampen innate responses to PAMPs in intestinal immune tissues to maintain intestinal homeostasis.

CHAPTER 6: TLR9 SIGNALING FAILURE RENDERS PEYER'S PATCH REGULATORY B CELLS UNRESPONSIVE TO CpG ODN STIMULATION

6.1 Abstract

Jejunal Peyer's patch (PP) regulatory $CD21^+$ B cells (B_{regs}) suppress TLR9-induced innate immune responses. However, TLR9 activation in B_{regs} has not been studied. Here, we investigated the responses of B_{regs} to stimulation with the TLR9 agonist, CpG ODN. B_{regs} express high levels of TLR9 mRNA, but fail to proliferate when stimulated with CpG. Furthermore, unlike $CD21^+$ B cells isolated from blood, B_{regs} do not secrete IgM or IL-12 following CpG stimulation. We hypothesized that the unresponsiveness of B_{regs} to CpG stimulation was due to an inability of the TLR9 receptor to activate the TLR signaling pathway. This was confirmed through kinome analysis which demonstrated dynamic patterns of phosphorylation of peptides representing TLR adaptor proteins such as IRAK1, TAK1, IKK, and NFκB-p65 in $CD21^+$ B cells from blood B cells but not B_{regs}. Therefore, a dysfunctional TLR9 signaling pathway may be an important mechanism by which mucosal B_{regs} fail to respond to TLR9 agonists.

6.2 Introduction

Toll-like receptors (TLRs) are the most studied family of pattern recognition receptors (PRR) and have been found to be important in the detection of microbial threats and play an essential role in the induction of immune responses [4, 18]. TLRs are expressed by cells of innate and adaptive immune system and recognize an array of pathogen-associated molecular patterns (PAMPs) such as double stranded RNA, LPS, single stranded RNA and bacterial DNA [41, 171-174]. TLR9 detects bacterial DNA or synthetic CpG ODN [41]. Activation of TLR9 by CpG ODN leads to recruitment of downstream adaptor molecules including MyD88, TRAF6, IRAK1, TAK1, IKKα, IKKγ, p-38, JNK and Fos, which subsequently trigger NFκB and/or AP-1. This leads to up-regulation of innate immune response genes resulting in production of proinflammatory cytokines, type I interferons and cell proliferation. In humans [92] and in ruminants [93], the main cell populations that express TLR9 and directly activated by CpG ODN are B cells and plasmacytoid dendritic cells (pDC).

Interestingly, several studies have found that B cells, which are adaptive immune cells, constitutively express TLRs mainly TLR1 and TLR6-10 [74, 297] . These TLRs are subsequently up-regulated when the B cells are activated by either CD40 ligation or BCR stimulation. B cell activation by the TLR9 agonist, CpG ODN, was first reported by Krieg and colleagues who found that *in vitro* and *in vivo* stimulation of murine B cells with CpG ODN induced cell proliferation and secretion of immunoglobulins [54]. Subsequently, CpG ODN has been shown to directly stimulate B cells to preferentially secrete 'Th1-like' immunoglobulins IgG_{2a}, IgG_{2b} and IgG_3 in mice [298] and also to secrete cytokines such as IL-6, IL-12 and IL-10 [197]. In humans, CpG ODN can induce class switching to IgG1, IgG2 and IgG3 but significant secretion of immunoglobulins requires additional signals such as BCR cross-linking, CD40 ligation and dendritic cell-derived B cell activating factors such as BAFF and APRIL [74, 299]. Additionally, CpG ODN seems to require cooperative stimulation from CD40L or signals from pDC to induce cytokines such as IL-6 and IL-10. However CpG ODN alone can induce costimulatory molecules and chemokine receptors on human B cells [78, 300]. There seems to be substantial species-specific differences with respect to

stimulation of B cells with CpG ODN. In mice, all sub-types of B cells seem to be directly activated by CpG ODN whereas in humans, direct activation occurs mainly in activated and memory B cells from blood.

We recently reported a novel B cell (B_{regs}) population with regulatory functions in sheep Peyer's patches (PP). These PP B_{regs} suppress IFNα, IFNγ , and IL-12 through IL-10 and possibly other mechanisms [301]. However, how TLR responses are regulated in these B_{regs} have not been explored. The objective of the present investigation was to determine whether PP B_{regs} can be activated by CpG ODN. Investigations at the level of both biological responses and phosphorylation-mediated signal transduction indicate that B_{regs} do not respond to stimulation with the TLR9 agonist, CpG ODN. The absence of apparent TLR9 signaling events immediately following stimulation indicates that signaling is blocked close to the receptor.

6.3 Materials and methods

6.3.1 Oligonucleotides and Animals

The sequences of the three oligodeoxynucleotides (ODNs) used in this study have been published (Table 3.1) [301]. B-class (2007) CpG ODN was obtained from Merial Limited (Lyon, France), C-class (2429) from Coley Pharmaceutical Group (Wellesley, MA) and control non-CpG ODN 2007GC was purchased from Operon (Alameda, CA). Synthetic single stranded RNA oligoribonucleotide (ORN) 1075 was obtained from Coley Pharmaceutical Group (Ottawa, ON, Canada).

Suffolk sheep of either sex (3 to 4 months of age) were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada) and were housed at the Vaccine and Infectious Disease Organization (VIDO) animal facility. All experiments were carried out according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

6.3.2 Isolation of PBMC, JPP, and lymph node cells (LN) and tissue culture conditions

Blood was collected from the jugular vein of sheep in ethylene di-amine tetra-acetic acid (EDTA)-treated vacutainer tubes (BD Biosciences, Mountain View, CA, USA) and PBMC were isolated using 54% isotonic Percoll^{TM} (Pharmacia Biotech AB, Uppsala, Sweden), as described previously [201, 212].

Sheep were euthanised and mesenteric lymph node (mLN) and jejuna Peyer's patches (JPP) were collected and cells isolated as described before [110, 245]. The number of viable cells was determined by trypan blue dye exclusion and counting in a hemocytometer under a light microscope. Cells were resuspended in AIM V medium containing 2% fetal bovine serum (FBS). For magnetic activated cell sorting (MACS) isolation, cells were resuspended in MACS buffer (PBSA, 0.5M EDTA and 10% BSA).

Stimulation of PBMC, JPP, and mLN cells was performed in 96-well, round bottom plates (Nunc, Naperville, IL, USA) using AIM V medium supplemented with 2% FBS, 100 IU/mL Penicillin, 100 µg/mL Streptomycin sulfate, 0.25 µg/mL Amphotericin B, 2 mM L-glutamine, 50 µM 2-Mercaptoethanol and 10 µg/mL Polymyxin B Sulfate (Sigma-Aldrich) as described before [301]. For each treatment, $5x10^5$ cells were cultured in triplicate wells in 200 µL total volume. Culture supernatants were harvested and stored at -20^oC until assayed for IL-10, IL-12 and IgM.

6.3.3 Enzyme-linked immunosorbent assay (ELISA) for IL-10, IL-12 and IgM

ELISA for quantifying cytokines in cell culture supernatants were performed as previously reported for IL-12 [214] and IL-10 [249]. For IgM ELISA, serum was diluted using PBS with 0.05% Tween 20 (PBST) and positive control (sheep pre-bleed serum) was serially diluted (1/10, 1/100, 1/1000, to 1/1000,000) while supernatants were serially diluted by 3-fold. Diluted samples were incubated for 2 hours and then washed. The captured IgM antibodies were detected in one step by using alkaline phosphatase labelled rabbit anti-sheep IgM antibody (KPL, MD, USA; Cat: 052303) and visualized by using p-nitrophenyl phosphate (PNPP).

6.3.4 Cell purification using magnetic activated cell sorting (MACS)

The B cell population was obtained by enriching for $CD21^+$ B cells. Routinely, purified cells were >94% pure for $CD21^+$ cell population. The $CD21^+$ B cells fraction of PBMC and JPP was isolated as previously described [246, 302].

6.3.5 Caspase assay

Caspase-3/7 enzyme activity was determined using Caspase-Glo 3/7 assay (Promega, Madison, USA) according to manufacturer's instructions. Briefly $CD21^+$ B cells were

isolated as described above and stimulated with media, CpG ODN (5 ug/ml), or GpC (5 ug/ml). After 48 hr of incubation, 100 µl of Caspase-Glo® 3/7 Reagent (Promega Corporation, USA) was added to each well of 96-well plate. Luminescence in each sample was measured using the plate-reading Luminometer (Victor^{3}V, PerkinElmer, Inc., CA, USA).

6.3.6 Lymphocyte Proliferative responses (LPR)

Cells were re-suspended in culture medium at 2.5 x 10^5 cells per well in a final volume of 200 µL. Triplicate cultures were stimulated and incubated as already described above. During the final 6 hrs of 72 hrs incubation, cells were pulsed with 0.4 µCi ^{3}H-Thymidine (Amersham Pharmacia, Piscataway, NJ). Cells were harvested using standard liquid scintillation protocols and uptake of ^{3}H-Thymidine was assessed in a beta counter (Topcount, Packard Instrument Company, Meriden, CT). Cell proliferation was calculated as the mean counts per minute (c.p.m.) of triplicate cultures and expressed as a stimulation index (c.p.m. in the presence of stimulus/c.p.m. in the absence of stimulus). For the LPR assay, a γ-irradiated cell line expressing murine CD40-ligand (CD154), a known ovine B cells mitogen [303, 304] was added at a 1:10 ratio to confirm that B cells in enriched cultures were capable of a proliferative response.

6.3.7 Quantitative RT-PCR

RT-PCR was used to quantify TLR9 mRNA in the JPP ($CD21^+$ and $CD21^-$) and PBMC ($CD21^+$ and $CD21^-$) population as described previously [251]. Samples were normalized internally using the average cycle threshold (Ct) of beta-actin (β actin) as a reference. Values were expressed as delta Ct value per 500 ng total input RNA.

6.3.8 Kinome Analysis

A description of the rationale and process of construction of the bovine specific peptide arrays for kinome analysis has been presented elsewhere [305]. Briefly, peptides were selected to represent phosphorylation events associated with a spectrum of cellular events but with emphasis on responses associated with innate immunity. Bovine consensus sequences of the human and mouse peptides selected from Phosphosite were obtained by employing the Blastp program from NCBI to compare collected human peptides against the NCBI bovine protein database. Blastp was set to retrieve short exact matches. Parsed Blastp^{SM} results revealed that the majority of the hit sequences had 100% identity to their query sequences and a comparison of the protein descriptions for the query and hit sequences confirmed that they referred to the same protein. Similarly, we used Blastp^{SM} to determine whether the array was also ovine specific. Analysis of the 16 peptide sequences (described in table 6.1) by Blastp$^{\mathbf{SM}}$ revealed that they are the same protein in sheep.

Isolated purified $CD21^+$ B cells (25 X 10^6) from JPP and PBMC were stimulated with medium alone or 5 µg/ml of 2429 CpG ODN for 4 hrs at 37^0C and 5% CO_2 and 95% humidity. Cells were pelleted and stored at -80 °C before use with the peptide arrays. Cell lysate was prepared and incubated with the arrays as reported previously with the exception that lysates from 25 x 10^6 $CD21^+$ B cells were incubated with each chip (10). Briefly, cell pellets were lysed with 100 µl lysis buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/mL leupeptin, 1 g/mL aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF)), incubated on ice for 10 minutes and then spun in a microcentrifuge for 10 minutes at 4 ºC. A 70 µl aliquot of this supernatant was mixed with 10 µl of the activation mix (50% Glycerol, 50 uM ATP, 60 mM $MgCl_2$, 0.05% v/v Brij-35 and 0.25 mg/mL BSA) and incubated on the chip for 2 hours at 37 ºC. Finally, slides were washed once with Tris-buffered saline (PBS) (1 x solution; pH 7.3) containing 1 % TRITON® X-100, twice with 2 M NaCl containing 1% TRITON X-100 and in demineralized H_2O. Phospho-protein specific stain peptide arrays were performed. Following incubation, slides were

washed once in PBS-Triton then submerged in stain (PRO-Q® Diamond Phosphoprotein Stain, Invitrogen) with agitation for 1 hour. Arrays were then washed in tubes containing destain [20% acetonitrile (EMD Biosciences, VWR distributor, Mississauga, ON) and 50 mM sodium acetate (Sigma) at pH 4.0] for 10 minutes three times with the addition of destain each time. A final wash was done with distilled water. Arrays were dryed and read using a GENEPIX® professional 4200A microarray scanner (MDS Analytical Technologies, Toronto, ON) at 532-560 nm with a 580 nm filter to detect dye fluorescence. Images were collected using the GENEPIX 6.0 software (MDS). Images were then loaded on ARRAYVISION® (Image Research Inc). Intensity values for the spots and background were obtained and normalization (80^{th} percentile per chip) and statistical analyses were performed with GENESPRING® (Agilent Technologies) software. We used four biological replicates and each slide contains triplicate array. The results were shown as fold change in phosphorylation and measured relative to the respective unstimulated $CD21^+$ B cells.

6.3.9 Statistical analysis

Data were analyzed using the statistical software GraphPad Prism 5 (Graphpad, San Diego, CA, USA). Statistical differences in median values between two groups were determined using the Kruskal-Wallis test and comparisons were done between groups using Dunn's multiple comparison test. Values of $p<0.05$ were considered significant.

6.4. Results

6.4.1 Peyer's patch cells proliferate poorly in response to CpG ODN stimulation.

We evaluated lymphocyte proliferative responses in JPP stimulated with CpG ODN and these responses were compared to those of mLN and PBMC. Both PBMC and mLN exhibited significantly higher proliferative responses ($p<0.05$) compared to JPP (Fig. 6.1). However, of note, the proliferative responses in PBMC were significantly higher than in mLN.

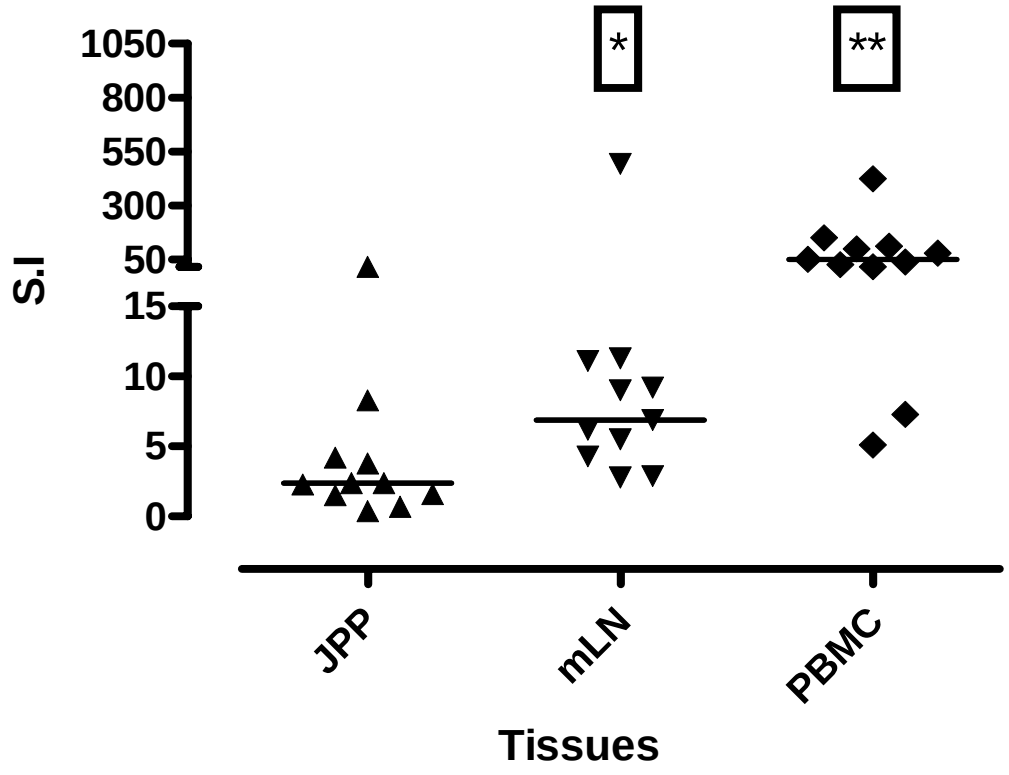

Fig. 6.1: Lymphocyte proliferative responses in jejunal Peyer's patches (JPP), mesenteric lymph node (mLN) and peripheral blood mononuclear cells (PBMC) stimulated with 2 µg/mL of CpG ODN 2429. *Significantly ($P<0.05$) higher than JPP.** Significantly higher than the other tissues.

Furthermore, we performed a dose titration experiment using CpG ODN doses of 0.66, 2.0, 6.0 and 18.0 μg/mL to test responses of PP over a wider dose range.

As shown in Fig. 6.2 A, in JPP, C-class (ODN 2429) induced significant proliferation of JPP cells at 6 and 18 μg/mL (Fig. 6.2 A). No significant proliferation was observed with the other ODN and regardless of the concentration of ODN used (Fig. 6.2 A).

In mLN, the optimal dose of CpG ODN was 2 μg/mL and increasing the concentration of ODN did not result in any additional response (Fig. 6.2 B). Similarly, in PBMC, 2 μg/mL of CpG ODN induced an optimal proliferative response and increasing the concentration of ODN did not result in any additional response (Fig. 6.2 C) with the exception of ODN 2336 (A-class). Therefore, the poor proliferative responses in PP following stimulation with CpG ODN are not due to suboptimal concentrations of the ODN.

In humans, purified blood B cells have been reported to respond to direct CpG ODN stimulation [92]. In this study, we wondered whether purified B cells from JPP would respond directly to CpG ODN stimulation in a similar fashion as blood B cells in sheep. Therefore we isolated $CD72^+$ cells (all B cells express CD72) using MACs from both PBMC and JPP and assessed their responses to CpG ODN. As previously demonstrated, PBMC proliferated significantly to CpG ODN (Fig. 6.3 A-B). Similarly purified blood $CD72^+$ B cells proliferated significantly to CpG ODN stimulation whereas blood $CD72^-$ cell fraction did not respond to either class of CpG ODN (Fig. 6.3 A-B).

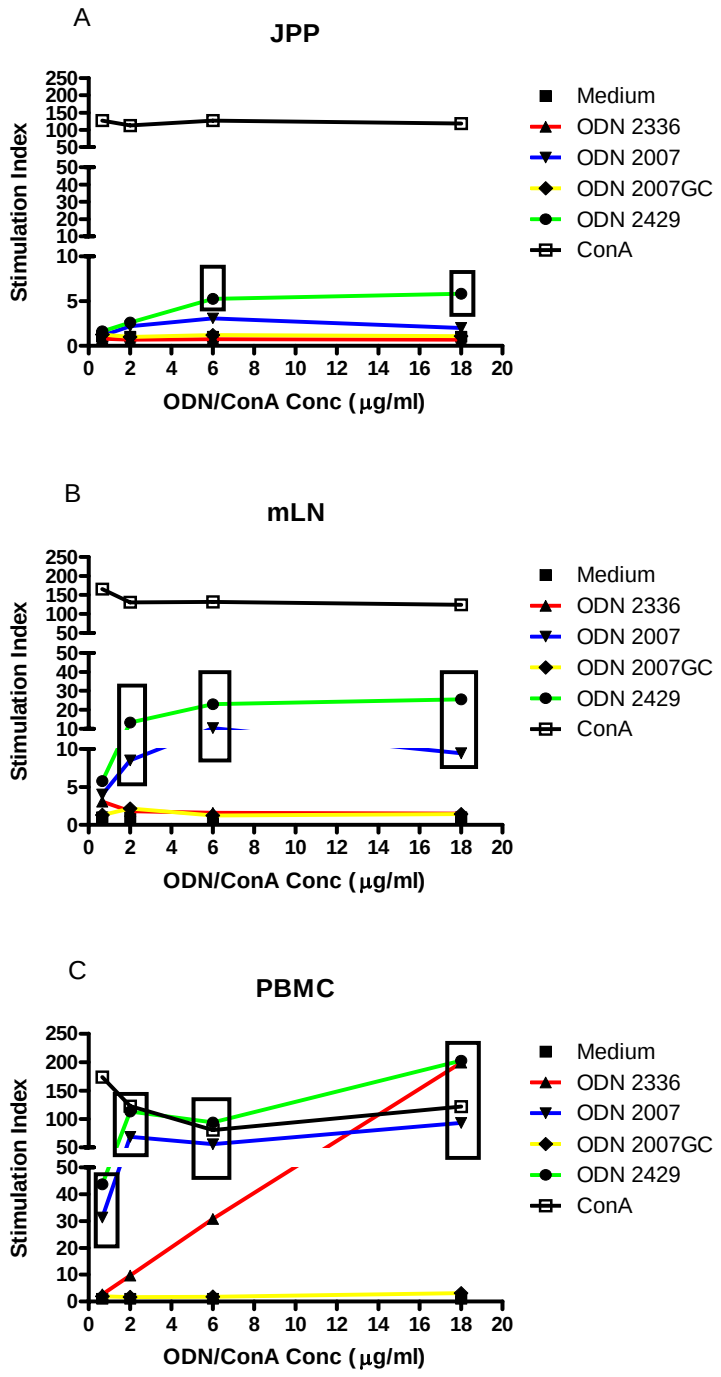

Fig. 6.2 A-C: Lymphocyte proliferative responses by ovine JPP, mLN and PBMC following stimulation with varying concentration of all 3 classes of CpG ODN. Data represent median value for cells isolated from 8 animals and assayed with each concentration of CpG ODN.

This observation suggested that CpG-induced proliferation in PBMC is a result of B cells proliferation. In contrast, JPP cells did not proliferate upon stimulation with CpG ODN and neither did the $CD72^+$ or $CD72^-$ B cell fractions (Fig. 6.3 A-B) following stimulation with both B- and C-class ODN.

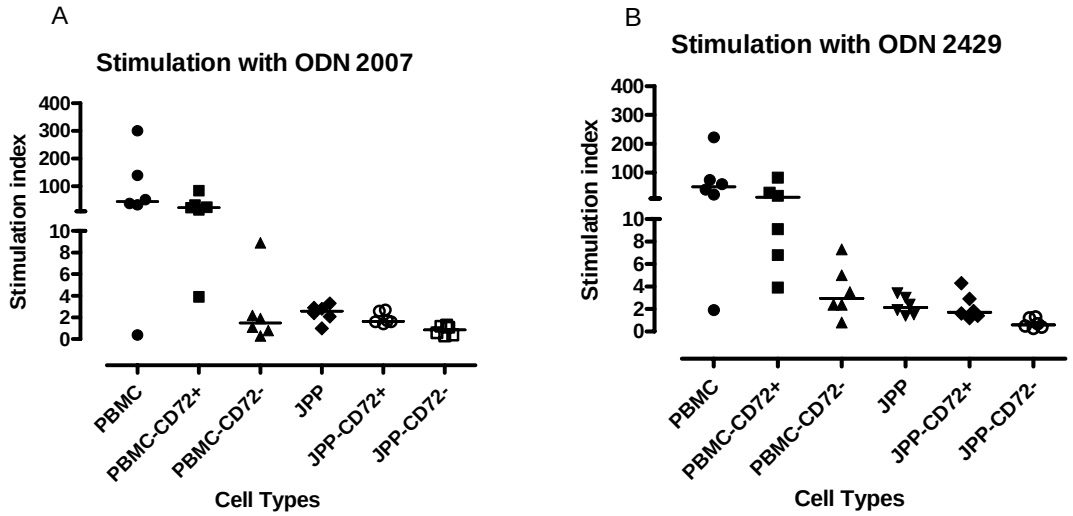

Fig. 6.3 A-B: Lymphocyte proliferative responses in JPP, PBMC, isolated $CD72^+$ and $CD72^-$ fractions from both tissues. A: Stimulated with 5 µg/mL of CpG ODN 2007.B: Stimulated with 5 µg/mL of CpG ODN 2429.

Sub-populations of B cells (naïve, activated and memory) have been reported to respond differentially to TLR activation in blood [51, 74, 75]. In the present study, blood $CD21^+$ B cells isolated from sheep were highly proliferative when stimulated with both classes of CpG ODN (Fig. 6.4 A-B) but the $CD21^-$ population had a much lower proliferation response (Fig. 6.4 C). In contrast, CpG ODN did not induce any significant proliferative responses in purified $CD21^+$ B cells from PP (Fig. 6.4 E). Therefore, $CD21^+$ B cells from blood and PP behave quite differently with respect to their proliferative responses to CpG stimulation. However, CD154 induced significant proliferation on PP $CD21^+$ B cells, indicating that these cells were viable and have a proliferative capacity when appropriately stimulated. Interestingly, the $CD21^-$ population in PP had a higher proliferative response upon stimulation by CpG ODN

(Fig. 6.4 F). This observation suggests that CD21$^+$ B cells downregulate CpG-induced proliferation in PP cells.

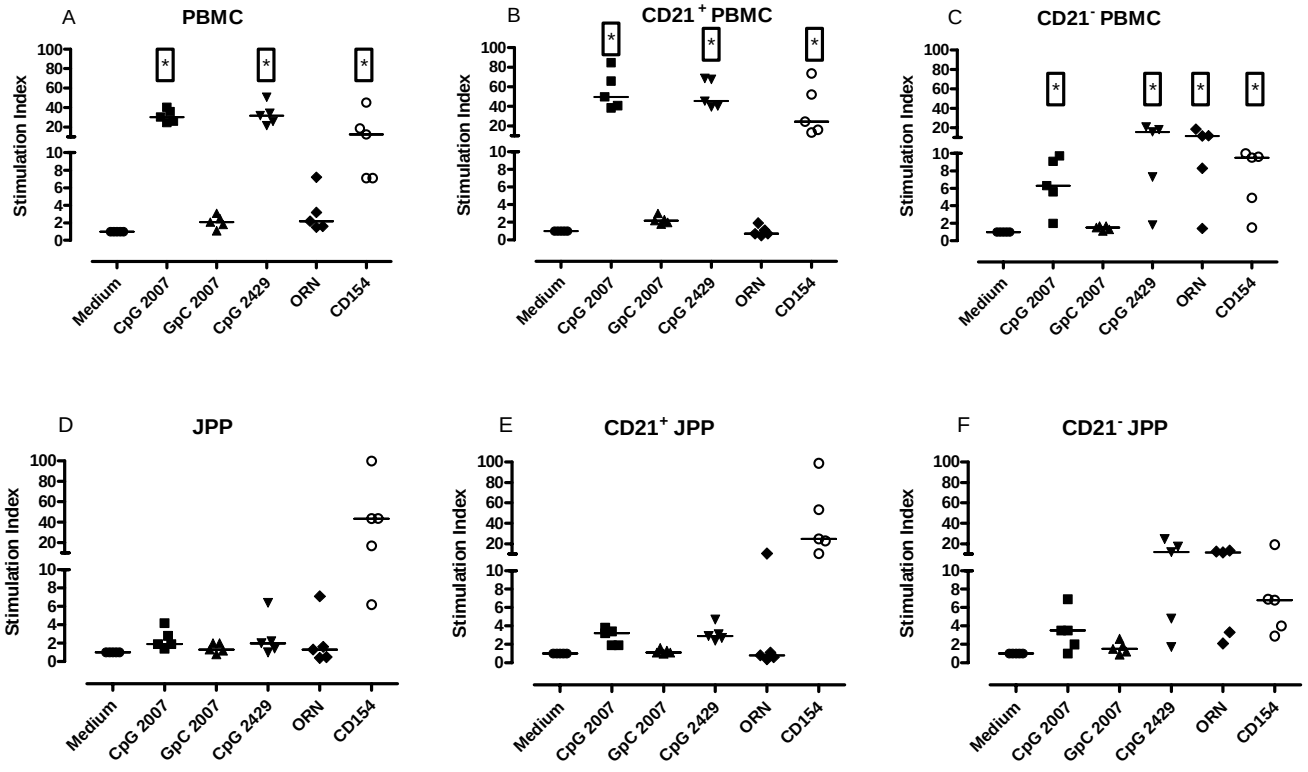

Fig. 6.4 A-F: Lymphocyte proliferative responses in JPP, PBMC, purified CD21$^+$ and CD21$^-$ cell fractions from both tissues stimulated with either medium alone, CpG 2007, GpC 2007, CpG 2429 or CD154.

6.4.2 Peyer's patches CD21$^+$ B cells fail to secrete significant IgM upon stimulation with CpG ODN.

B cells produce polyclonal IgM antibody following stimulation with CpG ODN [54]. We therefore assessed whether PP CD21$^+$ B cells secreted IgM in response to CpG ODN stimulation, and compared these responses to CD21$^+$ B cells from blood. As expected, PBMC secrete significant levels of IgM when stimulated with CpG ODN (Fig. 6.5 A). Purified blood CD21$^+$ B cells secreted high levels of IgM in response to CpG ODN stimulation but no significant IgM production was observed in the CD21$^-$ cell fraction (Fig. 6.5 A) in PBMC stimulated with CpG ODN. In contrast, the mixed

population of PP cells did not produce any significant IgM upon stimulation with CpG ODN (Fig. 6.5 B). Neither $CD21^+$ nor the $CD21^-$ cell fractions from the PP produced significant IgM responses following stimulation with CpG ODN (Fig. 6.5 B). Thus unlike $CD21^+$ B cells isolated from blood, $CD21^+$ B cells isolated from JPP did not produce IgM when stimulated with CpG ODN.

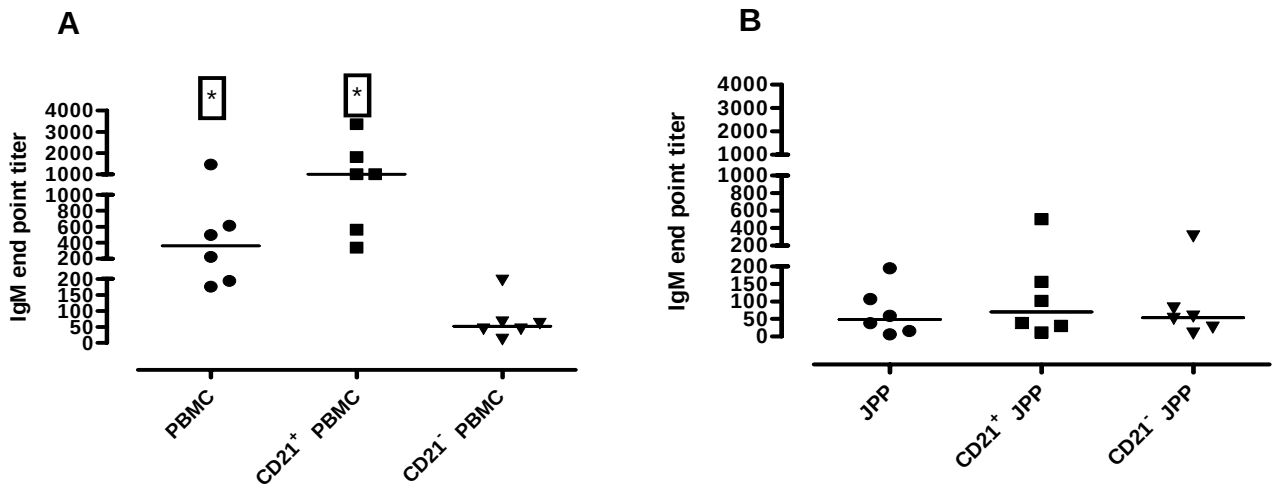

Fig. 6.5 A-B: IgM responses in JPP, PBMC, isolated $CD21^+$ and $CD21^-$ cell fractions from both tissues upon stimulation with 5 µg/mL of 2429 CpG ODN.

6.4.3 PP $CD21^+$ B cells express TLR9

One of the requirements for CpG ODN induction is the presence of its receptor, TLR9. We wondered whether the poor responsiveness of PP $CD21^+$ B cells to the CpG stimulation was due to a lack of TLR9 expression. Therefore we evaluated the expression of TLR9 in both PP and PBMC $CD21^+$ B cells using qPCR.

As shown in Fig. 6.6, both PP $CD21^+$ and blood $CD21^+$ B cells express similar levels of TLR9 mRNA. As expected both $CD21^+$ fraction expressed higher TLR9 mRNA compared to that of $CD21^-$ fraction. Thus the poor responses of PP $CD21^+$ B cells to CpG ODN stimulation may not be due to the lack of TLR9 receptor.

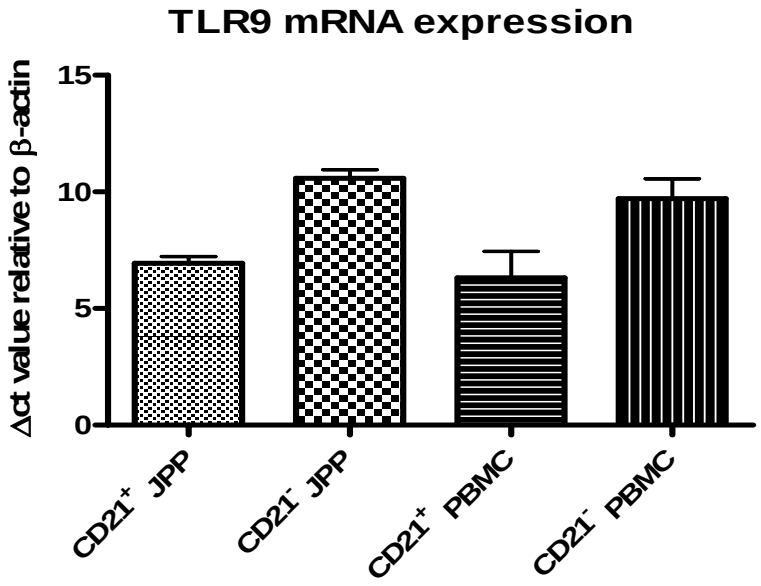

Fig. 6.6: TLR9 expression in JPP, PBMC, isolated $CD21^+$ and $CD21^-$ cell fractions from both tissues as assessed by qPCR. Results are normalized to beta-actin and total RNA used per sample was 500 ng.

6.4.4 PP and blood $CD21^+$ B cells express similar levels of apoptotic markers

One of the possible outcomes of CpG ODN stimulation in B cells can induce programmed cell death (apoptosis). Thus we evaluated whether stimulation of JPP $CD21^+$ B cells with CpG ODN induced apoptosis. Therefore we evaluated expression levels of apoptotic marker Caspase 3/7. We found that PP $CD21^+$ B cells had levels of Caspase 3/7 similar to those in $CD21^+$ B cells isolated from blood following stimulation with CpG ODN (Fig. 6.7). Therefore programmed cell death is unlikely to be the cause of the poor response in PP $CD21^+$ B cells stimulated with CpG ODN.

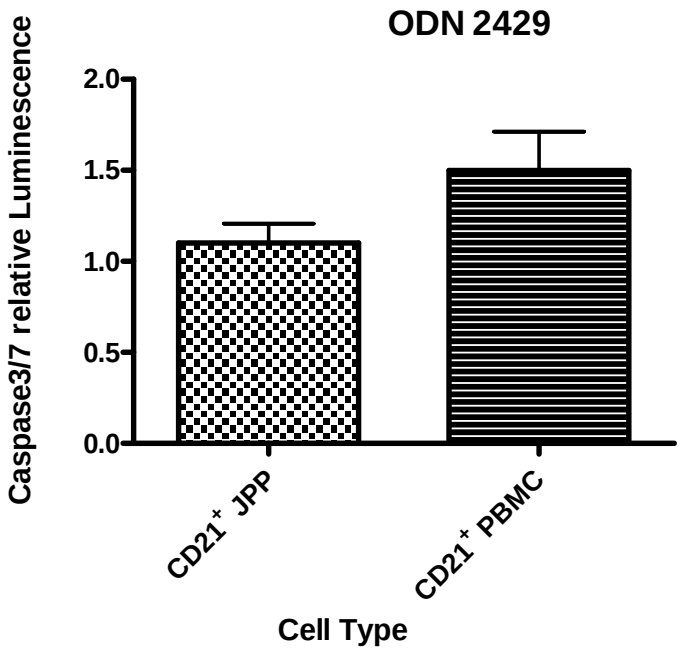

Fig. 6.7: Caspase 3/7 expression was assessed in JPP-CD21$^+$ and PBMC-CD21$^+$ cell fractions stimulated with 5 μg/mL 2429 CpG ODN or medium alone for 48 hrs. Results is expressed relative to medium alone treatment. Data represent the mean for 4 animals.

6.4.5 PBMC CD21$^+$ B cells but not PP CD21$^+$ B cells secrete IL-12

B cells are known mainly for the production of antibodies but they can also secrete a variety of cytokines including IL-12 when stimulated with TLR agonists [156]. Recently, it was shown that human and murine B cells secrete high levels of IL-12 when stimulated with CpG ODN and CD154 [160, 306]. Thus we tested whether CD21$^+$ B cells from blood and PP were capable of secreting IL-12 upon either CpG ODN stimulation or CD154. As shown by Fig. 6.8 A, purified PP CD21$^+$ B cells did not secrete IL-12 upon stimulation with either CpG ODN (C and B-class) or CD154. However, purified blood CD21$^+$ B cells secreted high levels of IL-12 in response to stimulation by both CpG ODN and CD154 (Fig. 6.8 B).

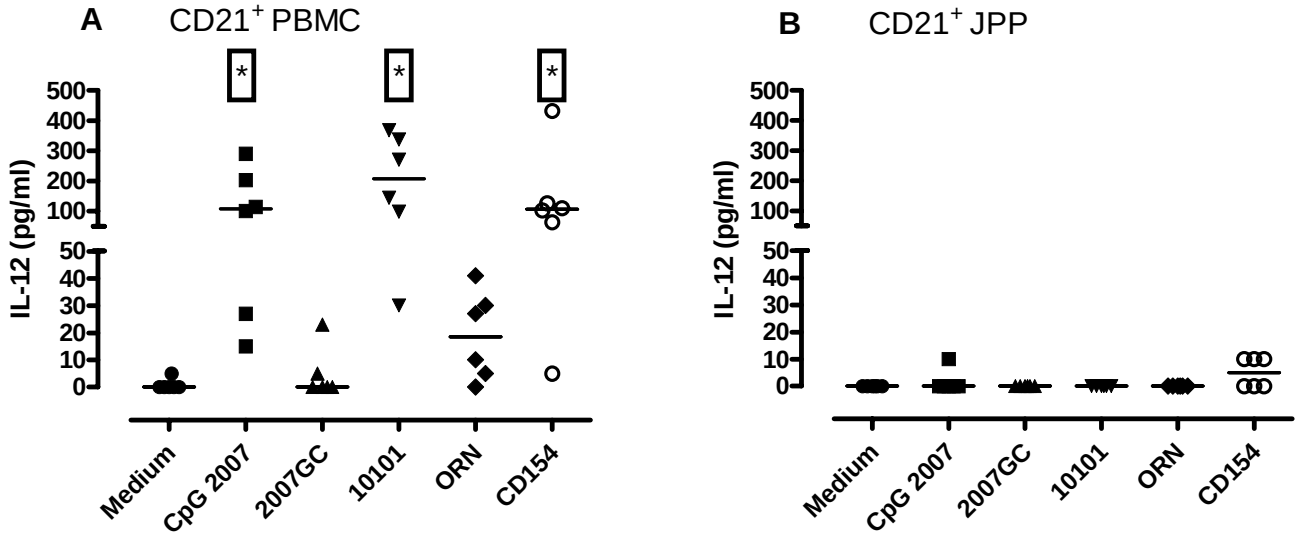

Fig. 6.8 A-B: IL-12 secretion in $CD21^+$ cell fractions from PBMC and JPP stimulated with 5 µg/mL 2429 CpG ODN, 2007CpG, CD154 or medium alone. Data represent the mean for 6 animals.

6.4.7 Unresponsiveness of PP $CD21^+$ B cells to CpG ODN is not due to IL-10.

Another potential cause of PP B cell failure to respond to CpG stimulation may be the influence of anti-inflammatory cytokines such as IL-10 and TGF-β [307]. IL-10 is known to have pleiotropic effects on B cells proliferation [136]. We assessed whether IL-10 had any effect on the responsiveness of these cells to CpG ODN. As reported previously (Chapter 5) [301], unstimulated purified $CD21^+$ B cells from PP spontaneously secrete IL-10. Therefore we tested whether neutralization of IL-10 had any effect on CpG-induced responses in PP $CD21^+$ B cells. Neutralization of IL-10 in PP $CD21^+$ B cell cultures did not result in any significant increase in proliferation following stimulation with CpG ODN (Fig. 6.9 A-B). Inhibition of IL-10 was also done using a P_{38} inhibitor (SB203580, a known inhibitor of IL-10 [308]) and similar results were obtained (data not shown). Thus IL-10 does not seem to play a role in the unresponsiveness of PP $CD21^+$ B cells to CpG ODN.

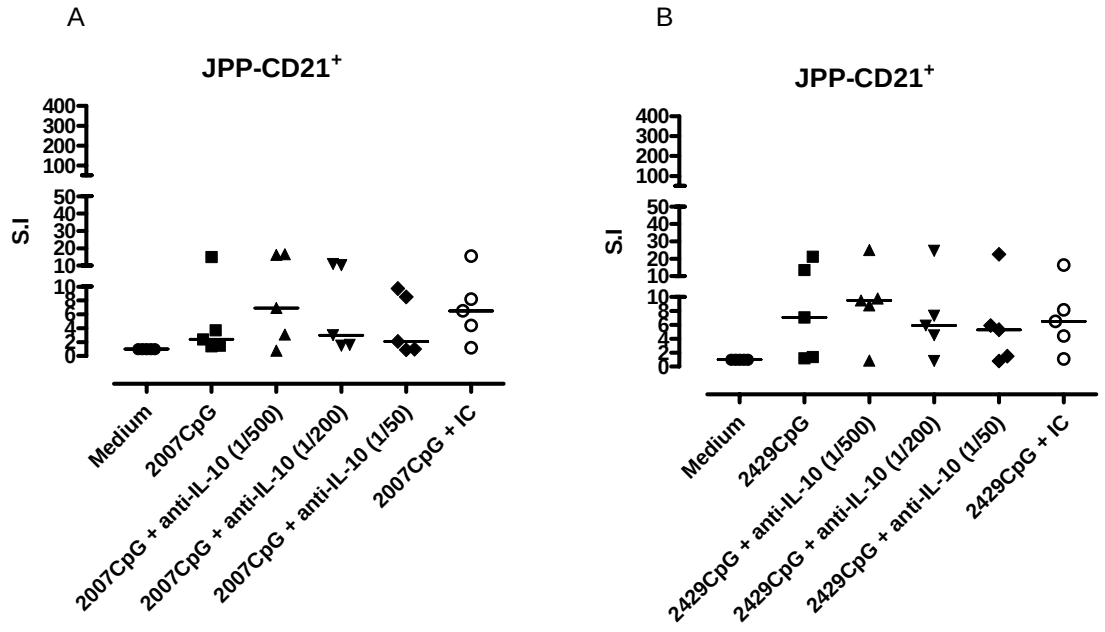

Fig. 6.9 A-B: Neutralization of IL-10 in PP CD21$^+$ B regulatory cells. Lymphocyte proliferative responses in PP CD21$^+$ B cells were assessed following addition of various concentrations of anti-IL-10 antibody and stimulation with 5 µg/mL 2007CpG (A), CpG2429 (B), isotype control (IC) or medium alone. Data represent the mean for 5 animals.

6.4.8 *Failure of TLR9 signaling in PP CD21$^+$ B cells*

Although CD21$^+$ B cells from blood and JPP express similar levels of TLR9 mRNA, there was a marked difference in B cell responses to CpG ODN stimulation. The seeming presence of the TLR9 receptor on both cell types suggests that the differential responses of these two cell types to CpG stimulation reflects unique patterns of signal transduction. To investigate this potential mechanism, kinome analysis was utilized to quantify levels of activity of kinases known to be activated in the TLR9 pathway. Both blood and PP CD21$^+$ B cells, with and without stimulation by CpG ODN were examined. PP and blood CD21$^+$ B cells showed marked differences in their overall signal patterns as well as numerous differences specific to TLR signaling. As shown in Table 6.1, sixteen out of the 300 peptides on the array revealed contrasting fold changes in phosphorylation when PP CD21$^+$ B cells were compared to blood CD21$^+$ B cells.

Table 6.1: Specific peptides on the array representing TLR9 adaptor molecules that are in stark contrast in $CD21^+$ B cells from JPP and PBMC in terms of their phosphorylation state.

TLR adaptor proteins	Target amino acid on the protein	JPP-$CD21^+$ CpG fold change	PBMC-$CD21^+$ CpG fold change	Effect of phosphorylation on target peptide [ref]
IKK-alpha	T23	-4.43	46	Activation [309]
Fos	T232	-113.6	2.13	Activation [310]
IKK-beta	Y199	54.2	1	Activation [311]
TAK1	S192	-67.37	72.2	Activation [312]
p38-alpha	Y323	-136.47	100.2	Activation [313]
JNK1	S377	-26.9	88.23	Activation [314]
TRAF6	T186	35.97	-13.1	Unknown
NFkB-p65	S276	1.02	5.25	Activation [315]
NFkB-p65	S311	-1.28	3.31	Activation [316]
NFkB-p65	S536	1.09	3.61	Activation [317]
IRAK1	T100	3.51	1.03	Inhibition [318]
IRAK1	T387	-3.82	9.11	Activation [319]
FADD	S194	-29.23	-1.36	Activation [320]
FADD	S194	-22.74	-1.25	Activation [320]
Casp8	S347	-6.18	4.34	Inhibition [321]
PKACa	T195/7	-7.76	3.62	Activation [314]
PKACa	S338	-6.27	1.12	Activation [322]

Activation of TAK1 depends on the phosphorylation of a series of C-terminal sites including Ser192 [323, 324]. This kinase target underwent 70 fold increase in phosphorylation in $CD21^+$ B cells from PBMC upon CpG ODN stimulation. In contrast, CpG stimulation of PP $CD21^+$ B cells induced a decrease (-50 fold) in phosphorylation of the same kinase target (Table 6.1). Activation of IKK-α also depends on phosphorylation of the amino acid Thr23 [309]. We found that upon CpG stimulation of blood $CD21^+$ B cells, this IKK-α target site had increased phosphorylation (46 fold) but stimulation of PP $CD21^+$B cells with CpG resulted in a decreased phosphorylation (-4.4 fold) of this peptide. Activation of p-38-alpha kinase depends on phosphorylation of target sites including Tyr323 [313]. Upon CpG stimulation of blood $CD21^+$ B cells, this target underwent 100-fold increase in phosphorylation, whereas PP $CD21^+$ B cells underwent a decrease in phosphorylation (-

136 fold) upon CpG stimulation. Similarly, JNK1 (S377), NFκB-p65 (S311, S276, and S536), FADD (S194), Casp8 (S347), PKACa (T195/7) and Fos (Thr232) have been shown to be activated when their respective targets are phosphorylated [314, 320-322, 325, 326]. We found that in blood $CD21^+$ B cells, all these targets displayed increased phosphorylation following stimulation with CpG ODN while in PP $CD21^+$ B cells, all those targets displayed decreased fold changes in phosphorylation (Table 6.1). The enzymatic activities of IRAK1 have been shown to be regulated differentially, that is phosphorylation of the T387 target site resulted in activation of the enzyme [319] while phosphorylation of the T100 target site resulted in inhibition of IRAK1 [318]. In blood $CD21^+$ B cells, we observed that IRAK1 (T387) was phosphorylated (9 fold higher) while in PP $CD21^+$ B cells, we found decreased (-3.8 fold) phosphorylation of IRAK1 (T387) following CpG stimulation. Interestingly, we observed in blood $CD21^+$ B cells, that the inhibitory IRAK1 target site T100 was unchanged in phosphorylation fold change (1.0) while in PP $CD21^+$ B cells, IRAK1 (T100) displayed increased phosphorylation (3.5 fold) upon stimulation with CpG ODN.

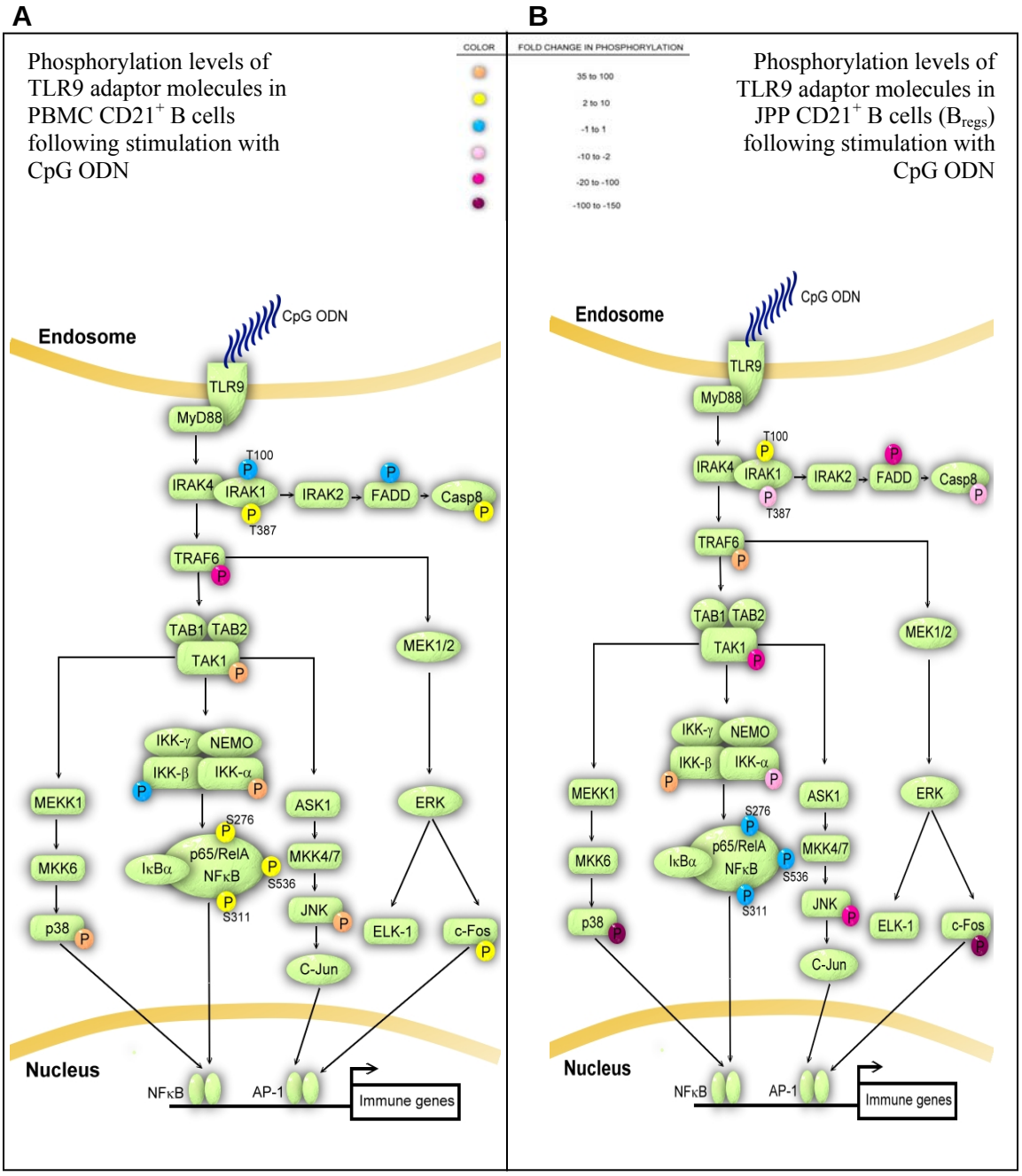

Fig. 6.10 A-B: Phosphorylation state of adaptor molecules in the TLR9 signaling pathway in CD21+ B cells isolated from (A) blood and (B) JPP. Color patterns indicate the fold change in phosphorylation.

As summarized in Fig 6.10 A-B, all of the above adaptor molecules are involved in the TLR9 pathway but they display differential phosphorylation fold changes in PP

compared to blood $CD21^+$ B cells upon stimulation by CpG ODN. These results demonstrate that the key adaptor molecules for TLR9 signaling transduction leading to NFκB, AP-1 and MAPK pathway activation are dysfunctional in PP $CD21^+$ B cells compared to blood $CD21^+$ B cells following stimulation with CpG ODN. Thus, the kinome data indicate that following CpG stimulation, PP $CD21^+$ B cells decrease phosphorylation of key adaptor molecules in the TLR9 signaling pathway.

6.5 Discussion

We previously observed that JPP B_{regs} have regulatory activity and down-regulate TLR9-induced IFNα, IFNγ and IL-12 responses, and this suppression is mediated at least in part through IL-10 [301]. In the present study, we demonstrated that JPP B_{regs} failed to proliferate, secrete IgM and produce IL-12 following CpG ODN stimulation.

We investigated the mechanisms for JPP $CD21^+$ B cells (B_{regs}) unresponsiveness to CpG ODN. The failure of B_{regs} to respond to CpG ODN was not due to a lack of TLR9 expression, CpG activation induced apoptosis or autocrine IL-10 regulation. However, CpG ODN unresponsiveness was not due to a lack of proliferative capacity in JPP B_{regs}, since vigorous proliferation was observed when these cells were stimulated with CD154 (a T cell signal). Thus, B_{regs} have the capacity to proliferate when appropriately stimulated.

PP B_{regs} demonstrated the hallmarks of the described B regulatory cells [152] as they exclusively produced IL-10 but not IL-12 even when stimulated with CpG ODN, ORN or CD154. Moreover, we demonstrated another regulatory aspect of B_{regs} as they suppress proliferation responses in other B cells. In this regard, depletion of $CD21^+$ B cells resulted in a significant increase in the proliferative responses of $CD21^-$ cell populations, presumably $CD21^-$ B cells. This result suggests that PP B_{regs} may regulate other B cells but the mechanisms by which they mediate this regulation are not known.

TLR9 is a requisite for CpG-induced responses [41]. We have shown that B_{regs} express similar levels of TLR9 mRNA as blood $CD21^+$ B cells, suggesting that an absence of TLR9 was not the cause of the unresponsiveness of PP B_{regs} to CpG stimulation. We then hypothesized that the failure of PP B_{regs} to respond to TLR agonists may be due to differential regulation of phosphorylation events downstream of the TLR9 signaling pathway. Therefore we used kinome analysis to characterize TLR9 signaling in PP $CD21^+$ B (B_{regs}) cells and blood $CD21^+$ B cells. Interestingly, in blood $CD21^+$ B cells which is highly responsive to TLR9 agonists, CpG ODN stimulation activates kinases such as IRAK1, TAK1, IKKα, p38-alpha, JNK1, FOS, NFκB-p65, FADD, Casp8 and PKACa which are associated with TLR9 mediated signal

transduction. In contrast, the above TLR adaptor molecules displayed a net decrease in phosphorylation in PP B_{regs} following CpG stimulation leading to inactivation of these kinases associated with TLR9 mediated signal transduction.

It seems that stimulation of PP B_{regs} cells by CpG ODN results in decreased phosphorylation of adaptor molecules early in the TLR9 pathway and causes inactivation of key adaptor molecules. Therefore, there may be regulatory mechanism in PP B_{regs} by which adaptor molecules are inactivated following ligand interaction with TLR9. Some key possible negative regulatory mechanisms in the TLR9 pathway that could be involved including: (i) factors that cause degradation of signal transduction molecules such as Triad3A (degrade specifically TLR4 and TLR9 receptor) [327], (ii) Inhibition of adaptor molecules such as SH2 homology 2 domain-containing protein tyrosine phosphatase (SHP)-1 (Inhibit IRAK1) [328], (iii) deubiquitination of adaptor molecules by factors such as A20 and deubiquitinating enzyme A (DUBA) [329],and (iv) non-functional TLR9 receptor. However, we have evidence that the TLR9 receptor is functional in PP B_{regs} under certain condition. When co-stimulated with CD154 and CpG ODN, B_{regs} proliferate significantly compared to stimulation with CD154 alone (Data not shown). This suggests that upon appropriate co-signaling, the TLR9 pathway can become active in PP B_{regs}. This may represent a novel way by which PP B_{regs} fail to respond to TLR agonists which are abundantly present in the gut.

6.6 Conclusion:

In conclusion, PP B_{regs} do not display biological responses to direct TLR stimulations despite expressing TLR9. However, kinome analysis confirmed TLR9 signaling did occur following CpG ODN stimulation. This response entailed differential signaling activity close to the receptor suggesting attenuation of both the primary and secondary TLR-induced signal transduction in PP B_{regs}. This raises important questions regarding the physiological role of the TLR9 receptor expression on this cell type.

CHAPTER 7: GENERAL DISCUSSION

7.1 Intestinal homeostasis

The intestinal tract represents the largest body surface that is in contact with the external environment. While the gut is an essential organ for the uptake of nutrients and fluids, most human pathogens enter the body through its mucosal surface. Normally, the intestine is continuously exposed to a large and diverse microbiota. In order to shape the intestinal environment, the mucosal immune system actively interacts with the microbiota, thereby limiting its level of penetration and growth in the gut lumen. However, when the intestine is exposed to dangerous pathogens, it elicits strong immune responses to confer protection to the host. Thus there is a very dynamic balance between the host protective immunity and regulatory mechanisms thereby establishing intestinal homeostasis.

The innate immune system (IIS) provides various mechanisms of defense that prevents microbes from gaining access to the apical surface of the IEC. For example, the apical side of the IEC is covered by a layer of mucus and glycocalyx which are produced by goblet cells present in the crypt and villus epithelium throughout the intestine [330]. The mucins trapped and allowed the elimination of commensals and pathogenic microbes by intestinal peristalsis [331]. Moreover, the IIS can produce a wide spectrum of antimicrobial agents that maintain the mucosal epithelial integrity [332].

In this thesis, we attempt to understand how the intestine regulates immune responses. We explored this issue by determining the responses of PP cells to PAMPs such as TLR agonists [CpG ODN, ORN, poly(I:C) and LPS]. In chapter four of this thesis, we demonstrated that the PP cells responded poorly to TLR agonists compared to other tissues (PBMC and LN). This hyporesponsiveness of PP cells could not be overcome by stimulation with combinations of TLR agonists, contrary to published reports with cells from blood. These results suggested that TLR responses are highly

regulated in PP cells. Therefore, this regulation in the PP must be achieved through regulatory mechanisms directed towards TLR agonists.

Peyer's patches are the primary sites for induction of antigen-specific immune responses. The intestine also expresses PRR including TLR receptors which detects PAMPs from microbes. We reasoned that understanding how TLR responses are regulated in PP may improve our understanding of how innate immune responses are regulated in the intestine. We therefore investigated the mechanisms which mediate the unresponsiveness of PP cells to TLR agonists. One of the mechanisms by which the intestine regulates innate responses is through the downregulation of innate receptors including TLR. Surprisingly, PP cells expressed similar levels of TLR7/8/9 as cells isolated from blood and LN. Thus PP cells respond poorly to TLR9 stimulation despite expressing TLR9 receptor.

The immunoregulatory cytokine IL-10 can modulate many different inflammatory pathways [136]. The involvement of IL-10 in the intestine has been shown through IL-10-knockout mice which tend to develop colitis in the presence of commensals [139]. Therefore, we explored whether IL-10 contributed to the unresponsiveness of PP to TLR agonists. As shown in chapter five, PP cells spontaneously secreted IL-10 which downregulated CpG-induced IFNα, IFNγ and IL-12 responses. In this regard, IL-10 was directly involved in the poor responses of PP cells to TLR9 stimulation. Moreover, cells that expressed the TLR9 receptor were able to respond to CpG ODN stimulation following neutralization of IL-10, suggesting that TLR9 receptors are functional in these PP cells. Subsequently, the IL-10-producing cells in PP were identified as $CD21^+$ B cells. Depletion of PP $CD21^+$ B cells resulted in significantly increased CpG-induced responses (IFNα, IFNγ and IL-12). These results demonstrate that PP $CD21^+$ B cells (B_{regs}) play a regulatory role in dampening TLR9-induced responses at least in part through the action(s) of IL-10. This finding has important implications in our current understanding of how immune responses are regulated in the intestine. B cells may develop a specialized regulatory role in the PP in order to actively lower immune responses to TLR agonists that are constantly present in the environment. Numerous studies have shown that DC and macrophages and even the epithelial cells have specialized functions in the intestinal environment [116, 127, 134].

Thus we report a novel B cell population capable of secreting IL-10 in the steady state, which may perform regulatory functions similar to the recently described murine B_{regs}.

7.2 B_{regs} : Characteristic and regulation of TLR9 signaling pathway

Usually, B cells are characterized by their ability to produce antibody. However, B cells have the ability to perform additional immune functions namely production of cytokines and to function as APC by taking antigen via their BCRs. Moreover, like T cells, there is a B cell population that can perform regulatory functions. B_{regs} were shown to develop in several murine models of chronic inflammation, including IBD, RA and EAE and they mediate their effects through the production of regulatory cytokines such as IL-10 and TGFβ [152]. In chapter 6, we showed that PP B_{regs} demonstrated the hallmarks of such described B regulatory cells [152] in much as they exclusively produced IL-10 but not IL-12 even when stimulated with CpG ODN, ORN or CD154. Further, the responses of the B_{regs} to CpG stimulation were tested. PP B_{regs} do not proliferate or secrete IgM following TLR stimulation alone. Whether B_{regs} secrete or regulate the production of IgA by other cells is unknown. However, we have an initial indication that B_{regs} may regulate the production of IgA in the PP. IgA has been reported however to play some roles in immune regulation in mucosal compartments [333].

Given that IL-10 plays a role in dampening innate responses, we explored the role of IL-10 in the seeming hyporesponsiveness of B_{regs}. Neutralization of IL-10 followed by CpG stimulation did not lead to B_{regs} proliferation but stimulation with CD154 led to the proliferation of these B_{regs}. These results suggest that B_{regs} are strictly regulated but may be activated under certain conditions.

Upon encountering their respective PAMPs, the TLR receptor is activated and initiates signal transduction pathways that culminate in potent transcriptional responses via NFκB. This is important in situations where immune responses are needed to rescue the host. However, inappropriate activation of the pathway may lead to constant inflammation. The intestine is constantly exposed to commensals expressing PAMPs. Thus there must be mechanisms by which the TLR signaling pathway is regulated. The

mechanism(s) by which cells from PP prevent overstimulation by PAMPs is unknown. There are several proposals on how the cells of the intestine could downregulate immune responses (reviewed in Honda and Takeda, 2009 [334]). Given the differences in TLR-induced responses between $CD21^+$ B cells isolated from PP and blood, we explored whether the TLR signaling pathway is functional in PP B_{regs} as compared to $CD21^+$ B cells from blood. In chapter six, we showed that there were major differences in the phosphorylation states of key adaptor molecules in the TLR9 pathway in PP B_{regs} compared to blood $CD21^+$ B cells. For example, adaptor molecules such as IRAK1, TAK1, FOS, IKK-α, JNK are not phosphorylated in PP B_{regs}. Thus the TLR9 pathway leading to activation of NFκB and AP-1 is dysfunctional in PP B_{regs}. These results suggest that there are negative regulators that keep adaptor molecules unphosphorylated in this cell type following CpG stimulation. The actual mechanism(s) that cause(s) negative phosphorylation in all these adaptor molecules remain unknown at this time and further investigations are required.

7.3 B_{regs}: Differentiating between commensals and pathogens

One of the fundamental questions in mucosal immunology is how the intestine differentiates between harmless commensal bacteria and pathogens. Numerous studies have revealed some of the regulatory mechanisms that maintain intestinal homeostasis (See chapter 1). For example, Lee *et al*, demonstrated that the IEC can distinguished between apical and basal TLR9 stimulation. Apical TLR9 stimulation led to unresponsiveness of the NFκB pathway whereas basal TLR9 stimulation resulted in hyperresponsiveness of the NFκB pathway leading to upregulation of cytokines [134]. Similarly, TLR5 which is the receptor for flagellin is located at the basolateral surface of the epithelium, thereby allowing it to recognize invading flagellated pathogens [335]. For example, commensal *E. coli* strains colonizing the apical surface of polarized model of intestinal epithelia do not activate pro-inflammatory gene expression whereas *Salmonella typhimurium* highly activate immune responses in these cells upon colonization. Therefore the compartmentalization of TLR has allowed the host to differentiate between pathogens and commensals. Similar mechanism could be used at

the cell level in B_{regs} where TLR9 may be expressed intracellularly in the endosome, lysosome and endoplasmic reticulum (ER). Thus the TLR9 agonist needs to be endocytosed and presented in the appropriate subcellular compartment for activation and signal transduction to occur. It has recently been reported that a 12-membrane spanning protein that resides in the ER, UNC93B1, is required for TLR7 and TLR9 trafficking [336]. In most cells, including $CD21^+$ B cells, the cellular location of TLR9 and requirement of trafficking proteins such as UNC93B1 for TLR9 function is unknown. Therefore regulation of TLR9 through subcellular compartmentalization may be one of the mechanisms involved in the gut.

It has been estimated that there are approximately 10^{14} bacteria in the gut. The microbiota is very diverse, comprising of about 500 species [337]. Therefore, commensals probably express most PAMPs, including TLR agonists, and therefore are similar to pathogens in this regard. However, pathogens differ from commensal bacteria by the presence of specific pathogenicity genes. These genes usually express virulence factors such as adherent molecules, invasion systems, enzymes and toxins. These virulence factors more importantly allow for pathogens to enter epithelial cells, invade and colonize the tissue, damage the host intestine and create a local or systemic infection. Therefore, not only TLR agonists will be present but also antigens and virulence factors from the pathogen would be available to trigger the host immune responses. Our observations that B_{regs} are not responsive to TLR stimulation indicate that there are regulatory mechanisms involved in the activation of these B cells. How mucosal B_{regs} differentiate between pathogens and commensals is still unknown. Below, we propose a model by which these cells could differentiate between pathogens and commensals.

7.3.1 Model

In the steady state, TLR agonists (from commensals) fail to induce robust immune responses in PP cells including DC, Mϕ, NK cells in the SED and B cells in the follicles. This is at least in part due to the IL-10 produced by $CD21^+$ B cells (B_{regs}) which subsequently downregulates other immune cells (presumably pDC, cDC and NK

cells) as suggested by our results. Furthermore, TGFβ is known to be produced in the intestine and contribute to regulation of inflammatory responses [338]. Thus, under the influence of mainly IL-10 and TGFβ, the PP maintain an "anti-inflammatory" state which allows for active regulation of immune responses to PAMPs including TLR agonists, and hence homeostasis. Moreover, the microenvironment around the follicles may be favorable for the generation of inducible iT_{regs} from $CD4^+CD25^-$ T cells since both TGFβ and IL-10 are present in the environment [307]. T_{regs} have already been proposed to contribute to the intestinal homeostasis. Therefore B_{regs} and T_{regs} together may contribute significantly to intestinal homeostasis in PP.

However, upon infection, the epithelial cells are breached and replicating pathogens invade the SED of the PP. The epithelial cells have been shown to respond to the breach by secreting cytokines and chemokines including IL-8 which will allow for the recruitment of cells from the systemic system. Therefore, high level of pathogenic molecules and TLR agonists are exposed to immune cells including DC in the PP. Resident and incoming DC can then be activated by both TLR and antigen and secrete pro-inflammatory cytokines such as IFNα and IL-12. Those DC would mature and migrate to the regional lymph nodes to present their cargo in terms of MHCII-antigen to local cognate T cells, thereby activating these cells, which would then migrate back to the gut and trigger cognate B cells. B_{regs} may also act as APC and sample the antigen and TLR agonists. It is not known whether these two signals are sufficient to activate B_{regs}. However, when they interact with cognate T cell help, B_{regs} are rapidly induced to proliferate as shown in the thesis. Together with the recognition of antigen through its BCR and by cognate T cell help and TLR activation, B_{regs} could produce specific antibodies against pathogenic antigens and could secrete proinflammatory cytokines such as IL-6, IL-12. Therefore, the microenvironment of the PP would thereby switch from an 'anti-inflammatory state into a pro-inflammatory condition. The strict requirement of T helper cells seems to be crucial for activation of PP B_{regs}. This may represent one of the mechanisms by which the intestine maintains homeostasis. Moreover, B_{regs} may only play a regulatory role without being activated to secrete antibody or other pro-inflammatory cytokines. However, this model is far from complete and many questions remain unanswered.

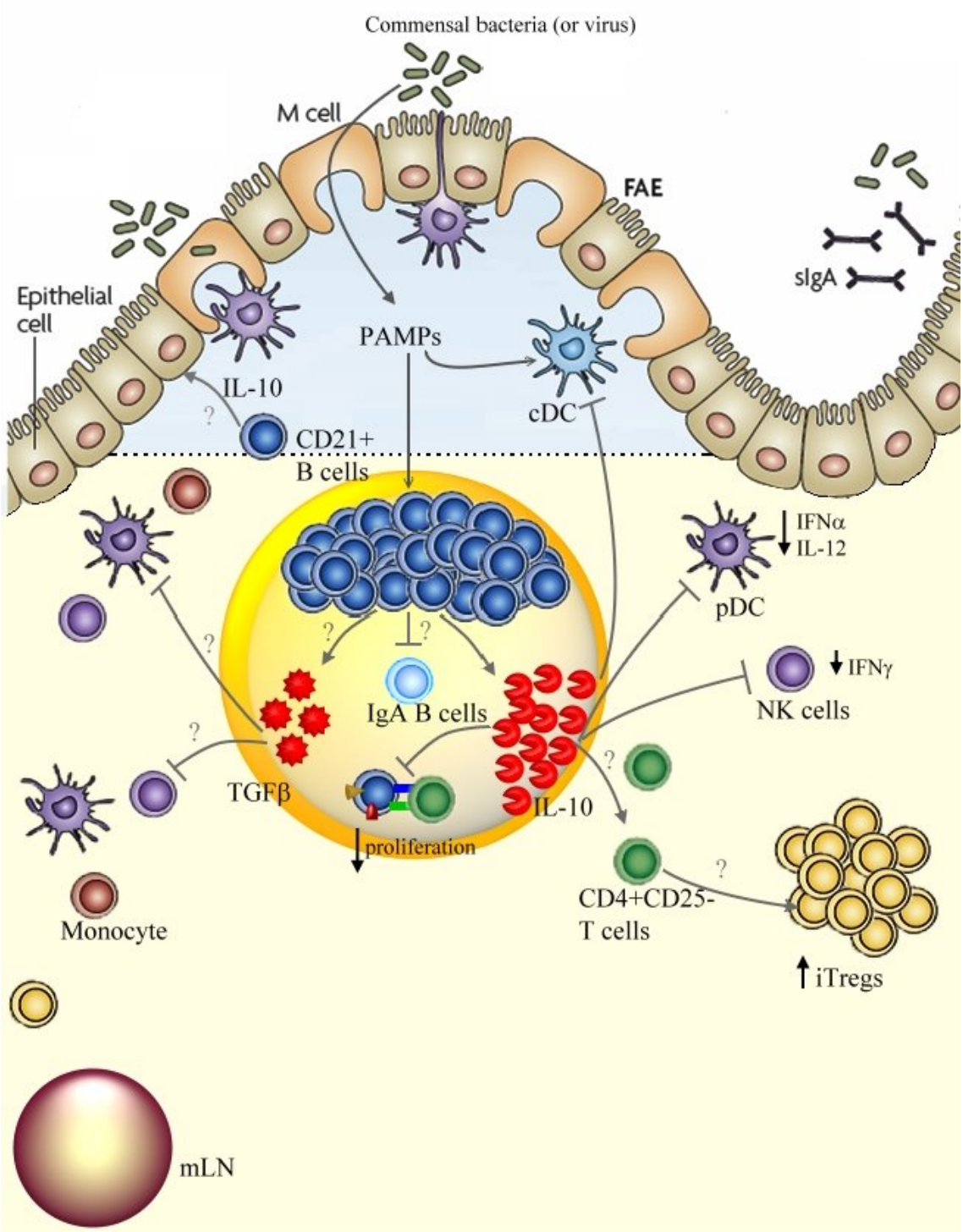

Fig. 7.1: Model demonstrating the potential role of PP B_{regs} in regulating TLR responses to commensal bacteria.

7.4 Significance of B_{regs} in Peyer's patches

In sheep, there are two distinct PP, namely the IPP and JPP. The IPP is a primary lymphoid organ that supplies the peripheral B cell pool in a manner similar to the way that the Bursa of Fabricus does in birds [100]. JPP are secondary lymphoid tissues that function in mucosal immune reactions similar to lymph nodes [110]. These two types of PP differ significantly in histology, ontogeny and lymphocyte trafficking [100]. In this thesis, we demonstrated that the JPP cells were significantly more responsive compared to the IPP cells following TLR stimulation. These results are consistent with the fact that JPP are primary sites for immune induction. It would make sense that downregulating TLR responses in JPP would be an effective way of avoiding unnecessary responses to commensals. We noted that in IPP cells, the levels of IL-10 expression were significantly higher than in the JPP and since IPP is not an immune induction site, we speculate that IPP may be a site for tolerance induction. However, the role(s) of IL-10 in the IPP is unknown at this time.

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CHAPTER 8: CONCLUSION

8.1 Overall Conclusion

In summary, we have shown that Peyer's patches respond poorly to TLR agonists despite expressing high levels of TLR mRNA. We clearly demonstrated that IL-10 plays a role in the reduced PP CpG-induced responses and that PP $CD21^+$ B cells (B_{regs}) spontaneously secrete IL-10 which "conditions" an anti-inflammatory environment in this tissue leading to poor cytokine responses to CpG ODN and presumably to other TLR agonists. This may represent a strategy by which PP dampen innate responses to PAMPs in intestinal immune tissues to maintain intestinal immune homeostasis. The regulation of TLR9 pathway in PP B cells seems to be achieved through the inactivation of multiple adaptor molecules in the pathway.

8.2 Future work

There are several directions that the work from this thesis can take. Of particular interest is the effect of B_{regs} on other B cells ($CD21^-$) including memory B cells that can be activated directly with TLR agonists. It would also be interesting to determine whether B_{regs} can have any role in the generation of T_{regs} or other regulatory cells. Several important questions remain unanswered. Do these B_{regs} exist in vivo? Are they restricted to PP or are they located in effector sites (e.g lamina propria) as well? Do they occur in other species or is this a phenomenon of sheep? Further investigations may elucidate how these B_{regs} work in the intestine.

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